Stress predisposition to necrotic enteritis caused by *Clostridium perfringens* in chickens and the administration of a complex microbiota to mitigate disease

by

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Abstract

Necrotic enteritis (NE) incited by the bacterium, *Clostridium perfringens*, is a disease of the small intestine of poultry that imparts a substantive economic cost and was previously controlled through the administration of in feed antibiotics. However, poultry production has entered an era of restricted antimicrobial use and this has prompted an urgent need for antimicrobial alternatives. Despite the best management practises, birds are exposed to unavoidable or unpredictable stressors that can influence various systems within the body. Limited research has examined the influence of physiological stress on the intestinal microbiota in poultry and its relationship to NE. Birds acquire many of their microbes from the environment and lack significant maternal transfer of microbes. Consequently, this results in a low diversity microbiota and renders birds susceptible to infections once exposed to pathogens on the farm. Administering a complex mixture of bacteria to birds shortly after hatch may promote colonization resistance in the intestine against incoming pathogens and expediting immune development in chicks to prevent future infections later in life. The objectives of my thesis research were: (1) elucidate the influence of stress on bird health; (2) examine the impact of stress on NE in layer chickens; (3) ascertain the impact of stress on NE in broiler chickens; and (4) determine the ability of a complex microbiota (CM) administered to day-old broiler chicks to ameliorate stress-induced NE. For all four objectives, physiological stress was incited by orally administering the glucocorticoid stress hormone, corticosterone (CORT) via water or feed. For objectives 2, 3, and 4, birds were challenged with a virulent strain of *C. perfringens* (CP1). For *Objective 1*, birds administered CORT exhibited altered lipid metabolism in the liver, and altered metabolite profiles in liver, kidney, and muscle. Moreover, stress altered the α- and β-diversity of bacterial communities within the small intestine and ceca. Notably, birds administered CORT possessed higher densities of indigenous *C. perfringens*. For *Objective 2*, layer chickens administered CORT and challenged with *C. perfringens* CP1 manifested subclinical NE. In this regard, body weight gain was reduced only in birds administered CORT and inoculated with *C.*

perfringens. Moreover, small intestinal mucus glycans were altered by *C. perfringens* colonization. In birds administered CORT, genes encoding tight junction proteins and toll-like receptors in the intestine were altered, as were inflammatory responses in the thymus and spleen. The administration of CORT also increased densities of *C. perfringens* in the small intestine. For *Objective 3*, broiler chickens administered CORT and challenged with *C. perfringens* manifested clinical NE. Birds challenged with *C. perfringens* showed higher histopathological scores; however, only birds administered CORT and challenged with *C. perfringens* exhibited gross necrotic lesions in the small intestine. *Clostridium perfringens* densities were highest in birds administered CORT, which in conjunction with increased pathological scores, indicated that stress is a predisposing factor to NE. For *Objective 4*, the model of NE from *Objective 3* was used to ascertain the degree to which the administration of a CM to day-old chicks influences NE. The CM was derived from cecal digesta of healthy broiler breeder adults, and was propagated in bioreactors. Birds administered the CM did not exhibit clinical signs of NE upon challenge with CORT and *C. perfringens*. Disease resistance was associated with an increased α-diversity and altered β-diversity of bacterial communities in the small intestine and ceca of birds administered the CM. Furthermore, birds that developed clinical NE exhibited decreased short-chain fatty acid production in the ceca, and this corresponded with a reduction in butyrate-producing bacteria in diseased birds. Evidence indicated that stress promoted disease by impairing immune and epithelial responses (e.g. *TLR2A*, *MUC2B*, *CATH1*) in the small intestine. Moreover, birds administered the CM counteracted responses to stress via stimulation of responses to fight infection and promote recovery (e.g. upregulation of *IL2*, *IL22*, *IL17*). Collectively, this thesis demonstrated physiological stress imparts alterations to bird metabolism, immune function, and the intestinal microbiota in a manner that contributed to increased susceptibility to NE. Moreover, the administration of a complex bacterial microbiota to day-old birds provided resistance to NE by increasing microbial diversity and promoting positive host responses.

Preface

The thesis is an original work by Sarah J. M. Zaytsoff. All research studies involving animals received ethics approval from the Animal Care Committee at the Lethbridge Research and Development Centre in Lethbridge, Alberta for the following projects: "Stress impacts on the host-microbiota interaction in chickens" ACC Protocol 1526, Nov 2015- Aug 2016; "Induction of necrotic enteritis caused by *Clostridium perfringens* and physiological stress" ACC Protocol 1707, May 2017- Feb 2018; and " Mitigation of stress-induced necrotic enteritis in broiler chicks via administration of a complex microbiota" ACC Protocol 1912, Apr 2019- Apr 2020.

Chapters 2, 3, and 4 of this thesis have been published. Chapter 2 has been published in Scientific Reports: Zaytsoff, S.J.M., Brown, C.L.J., Montina, T., Metz G.A.S, Abbott W.A., Uwiera R.R.E., Inglis G.D. Corticosterone-mediated physiological stress modulates hepatic lipid metabolism, metabolite profiles, and systemic responses in chickens. *Sci Rep* 9**,** 19225 (2019). I was responsible for executing experiments, analysis, and drafting the manuscript with the exception of the metabolomics component and histopathological scoring. Metabolomics analysis was conducted by T. Montina and C.L.J. Brown; and histopathological scoring was completed by R.R.E. Uwiera. Chapter 3 has been published in Microorganisms: Zaytsoff, S.J.M.; Uwiera, R.R.E.; Inglis, G.D. Physiological Stress Mediated by Corticosterone Administration Alters Intestinal Bacterial Communities and Increases the Relative Abundance of *Clostridium perfringens* in the Small Intestine of Chickens. *Microorganisms* 2020, *8*, 1518. I was responsible for executing experiments, analysis, and drafting the manuscript. Chapter 4 had been published in Gut Pathogens: Zaytsoff, S.J.M., Lyons, S.M., Garner, A.M., Uwiera R.R.E., Zandberg W.F., Abbott D.W., Inglis G.D. Host responses to *Clostridium perfringens* challenge in a chicken model of chronic stress. *Gut Pathog* 12**,** 24 (2020). I was responsible for executing the experiments, analysis, and drafting the manuscript with the exception of the mucin carbohydrate analysis and histopathological scoring. Mucin carbohydrate analysis was conducted by W.F. Zandberg, S.M. Lyons, and A.M. Garner; histopathological scoring was completed by R.R.E. Uwiera. Funding for these studies was obtained by G.D. Inglis. All co-authors contributed to manuscript editing and revisions.

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AAFC	Agriculture and Agri-Food Canada
ACC	Acetyl-CoA Carboxylase
ALDEx2	ANOVA-like Differential Expression 2
ANOVA	Analysis of Variance
AP-1	Activator Protein 1
APO	Apolipoprotein
APR	Acute Phase Response
APTS	8-aminopyrene-1,3,6-trisulfonate
ASV	Amplicon Sequence Variant
AvDB6	Avian Beta Defensin 6
BLAST	Basic Local Alignment Search Tool
CATH1	Cathelicidin 1
CBP	CREB Binding Protein
CCLi	Chemokine-like
CE	Capillary Electrophoresis
CE-LIF	Capillary Electrophoresis with Laser Induced Fluorescence
CFU	Colony Forming Unit
CL2	Containment Level 2
CLD	Claudin
CM	Complex Microbiota
CON	Control
CORT	Corticosterone
CP	Ceruloplasmin
DMBA	1,2-diamino-4,5-dimethylbenzene
ECC	Ethanol Carrier Control
FAS	Fatty Acid Synthase
FMT	Fecal Microbiota Transplant
G6Pase	Glucose-6 Phosphatase
GC-GR	Glucocorticoid bound to Glucocorticoid Receptor
G-CSF	Granulocyte-Colony Stimulating Factor

GLUT	Glucose Transporter
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
GRE	Glucocorticoid Response Element
H&E	Hematoxylin and Eosin
H:L	Heterophil to Lymphocyte ratio
HAT	Histone Acetylase
HDC / HD-C	High Dose Corticosterone (30 mg/L)
HMGCR	3-hydroxy-3-methyl-glutaryl-coenzymes A reductase
HPA	Hypothalamic Pituitary Adrenal
HPLC-MS	High-Performance Liquid Chromatography-Mass Spectrometry
HSP	Heat Shock Protein
IFNɣ	Interferon gamma
IGF-I	Insulin-like Growth Factor I
iNOS	inducible Nitric Oxide Synthase
Interleukin	IL
KEGG	Kyoto Encyclopedia of Genes and Genomes
LDC / LD-C	Low Dose Corticosterone (10 mg/L)
LeRDC	Lethbridge Research and Development Centre
LIF	Laser-Induced Fluorescence
LXR	Liver X Receptor
ME	Malic Enzyme
MLCK	Myosin Light Chain Kinase
MTTP	Microsomal Triglyceride Transferase Protein
MUC	Mucin
NAFLD	Non Alcoholic Fatty Liver Disease
NE	Necrotic Enteritis
NetB	Necrotic Enteritis β-like Toxin
NF-κB	Nuclear Factor kappa light chain enhancer of B cell
NMR	Nuclear Magnetic Resonance
OCLN	Occludin
OPLS-DA	Orthogonal Partial Least Squares Discriminant Analysis
p.h.	post-hatch

p.i.	post-inoculation
PEPCK	Phosphoenolpyruvate Carboxykinase
PERMANOVA	Permutational Multivariate Analysis of Variance
PICRUSt2	Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2
PLS-DA	Partial Least Squares Discriminant Analysis
PRR	Pattern Recognition Receptor
QIIME2	Quantitative Insights Into Microbial Ecology 2
qPCR	quantitative Polymerase Chain Reaction
ROC	Receiver Operating Characteristics
SAA	Serum Amyloid A
SAS	Statistical Analysis Software
SCFA	short-chain fatty acid
SPE	Solid Phase Extraction
SREBF1	Sterol Regulatory Element Binding transcription Factor 1
TEER	Transepithelial Electrical Resistance
TF	Transferrin
TGFβ2	Transforming Growth Factor β2
TJP1	Tight Junction Protein 1
TLR	Toll-like Receptor
TMSP	Trimethylsilylpropanoic acid
TNFα	Tumor Necrosis Factor alpha
VIAVC	Variable Importance Analysis based on random Variable Combination
VLDL	Very Low Density Lipoprotein

Chapter 1: Literature review

1.1 Introduction

Necrotic enteritis (NE) incited by the bacterium, *Clostridium perfringens*, is a disease of the small intestine of poultry that results in poor production performance, and imparts a substantive economic cost of $6 billion globally [1]. The addition of antibiotics in feed has longed been used as a control measure to prevent NE and other diseases. However, poultry production has entered an era of restricted antimicrobial use which has prompted an urgent need for antimicrobial alternatives. Management practises are being considered as an important measure to prevent NE [2]. Despite the best management practises, birds are exposed to unavoidable or unpredictable stressors that can influence various systems within the body and potentially predispose birds to NE. Physiological stress in birds activates the hypothalamic-pituitary-adrenal (HPA) axis and results in the production of the glucocorticoid, corticosterone (CORT) [3]. Glucocorticoids impart significant changes to a bird's metabolism and immune function which leads to poor production performance and increases the risk of disease [4]. Limited research has examined the influence of physiological stress on the intestinal microbiota in poultry and its relationship to NE. Poultry are unique from mammalian species as they acquire their first microbes from their eggshell and the hatchery, which is a relatively hygienic environment [5]. Consequently, this results in poultry containing a low diversity microbiota, which may render them susceptible to infections once exposed to pathogens on the farm [6]. Currently, strategies to discovering antimicrobial alternatives includes administering various products (i.e. essential oils) as a non-specific shot-gun approach. A drawback to these methods is the limited data on efficacy of the products and the mechanisms involved are unknown. Thus, research needs to be directed at providing proof-of-concept information on antimicrobial alternatives, including information on mechanisms of action. This not only provides important information for bird health, but can also potentially identify processes within a host that can be used to refine or create novel alternatives.

Microbiota transplants in humans have shown efficacy in preventing some diseases and infections [7], however, risks to this treatment have been identified. Birds present a unique challenge as they acquire many of their microorganisms from the environment and have limited maternal transfer of microbes [8]. Administering a complex mixture of bacteria to birds shortly after hatch may promote colonization resistance in the intestine against incoming pathogens and expediting immune development in chicks to prevent infections later in life. Examining microbiota transplants in poultry is needed from the "proof-of-concept" perspective. In this regard, not only does efficacy need to be measured, but deciphering the mechanisms involved on how microbiota transplants work to prevent

disease is also required [9]. This thesis reports on research that examined the impacts of physiological stress imparted on chickens with respect to metabolism, immune function, and microbiota development. Birds were administered a complex mixture of bacteria shortly after hatch to determine if NE induced by *C. perfringens* and stress could be mitigated. Various 'omics' and molecular strategies were utilized to achieve a mechanistic rationale-based solution to antimicrobial alternatives.

1.2 Stress and bird health

1.2.1 Molecular mechanisms of glucocorticoids

1.2.1.1 The glucocorticoid receptor

The glucocorticoid receptor is expressed in all cell types and contains three main domains: a transactivation, DNA binding, and ligand binding domain [10, 11]. The glucocorticoid receptor is found in the cytosol of cells in its non-active state, where it is bound to two heat-shock proteins (HSP90) [11]. These heat-shock proteins act as molecular chaperones to prevent nuclear localization [11]. Due to the lipophilic nature of glucocorticoids, they can pass freely through the plasma membrane and bind to the glucocorticoid receptor to facilitate activation [10]. Ligand binding to the glucocorticoid receptor elicits a conformational change that releases the HSP90, dimerizes, and then translocates to the nucleus [10, 11]. The glucocorticoid-receptor complex binds to glucocorticoid response element (GRE) sequences, which are located upstream of the promoter regions and can influence the transcription of various response genes [11]. This is considered to be the conventional mechanism of glucocorticoid action. It is now known that the ligand-bound glucocorticoid receptor (GC-GR) can influence the access of transcription sites by modifying chromatin structure, and exhibits non-genomic effects through protein-protein interactions with other transcription factors or signaling modules [10, 11]. The glucocorticoid receptor modulates physiological responses through a number of cellular events that result in the repression of some responses, while activating others.

1.2.1.2 Actions of the glucocorticoid receptor

(i) Repressive Effects to Transcription. Various cellular responses can be repressed by the ligand-bound glucocorticoid receptor by altering transcription or protein-protein interactions [10]. The efficacy of Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and Activator Protein 1 (AP-1) via activation of their transcriptional targets can be suppressed through direct interactions with GC-GR, including competition with GC-GR for the same transcriptional machinery [12, 13]. This results in inhibition of expression of cytokine genes, which can include interleukin (IL)-1β, tumor necrosis factor alpha (TNFα), granulocyte-macrophage colony-stimulating-factor (GM-CSF), IL-4, IL-5, and IL-8 [11]. The GC-GR can also repress genes through histone modifications. In this regard, high concentrations of the

potent glucocorticoid, dexamethasone, are capable of inhibiting histone acetylation necessary for inflammatory gene transcription, and also can induce histone deacetylase activity [14]. The prevention of histone acetylation results in chromatin retaining a wound structure and prevents transcriptional machinery from assembling [14]. The signaling protein, CREB binding protein (CBP), has histone acetylase (HAT) activity, which can facilitate various transcription factor binding, such as NF-κB and AP-1 [11]. The GC-GR may compete with NF-κB and AP-1 for binding to CBP and further promote the repression of gene transcription [11].

(ii) Activation Effects of Glucocorticoids. The GC-GR can activate many responses to alter physiological and immunological responses. One example is the activation of inhibitors that block inflammatory signaling. In this regard, dexamethasone administration to cells in culture results in increases in IκBα, which is an inhibitor of NF-κB, preventing its nuclear localization [15]. The GC-GR has been shown to upregulate several anti-inflammatory proteins such as lipocortin-1, secretory leukocyte protease inhibitor, and IL-1 receptor agonist [11]. Lipocortin 1 is inhibitory to phospholipase A2, which in turn inhibits the production of inflammatory mediators such as leukotrienes and prostaglandins. In addition to activating anti-inflammatory responses, the GC-GR can upregulate various factors involved in energy homeostasis. These include upregulation of gluconeogenic and lipogenic pathways in the liver, and genes involved in muscle atrophy [16, 17]. The activating mechanisms of the GC-GR complex are facilitated in the same manner as repressive effects ─ through DNA binding, protein-protein interactions, and interference to transcription by histone modifications [10].

1.2.2 Impacts of glucocorticoids to metabolism

1.2.2.1 Glucose metabolism in mammals

Endogenous glucose production occurs during times of nutrient deficiency to maintain stable glucose levels for tissues, brain function, and erythrocyte activity [16]. Glucocorticoids promote the production of glucose in the liver by modulating gluconeogenic processes [16]. Evidence shows the GC-GR complex mediates its effects on glucose homeostasis through DNA-binding to the promoter region of gluconeogenesis genes [16]. Studies in mice with a glucocorticoid receptor unable to bind DNA have demonstrated absence of upregulation of phosphoenolpyruvate carboxykinase (PEPCK), a key metabolic enzyme involved in the commitment to gluconeogenesis [18]. Furthermore, adrenalectomized mice show impairment of glucose 6-phosphatase (G6Pase) expression, an enzyme that completes the final step of gluconeogenesis and controls hepatic release of glucose from glycogenolysis [19]. The promoter regions of both PEPCK and G6Pase contain binding sites for the GC-GR complex [16, 20]. The DNA-binding effects of the GC-GR do not act alone to mediate gluconeogenesis [16]. The liver X receptor

(LXR) is a nuclear receptor that can modulate the recruitment of the glucocorticoid receptor to gluconeogenesis promoters [21]. For example, rats treated with LXR agonists were protected from glucocorticoid induced hyperglycemia [21]. The LXR may compete with the GC-GR complex for binding the gluconeogenesis promoter [21]. Overall, mammalian research has provided a more mechanistic understanding of glucocorticoid actions on molecular functions and this information was often contributed with the use of murine transgenic and knock-out models.

1.2.2.2 Glucose metabolism in poultry

In general, changes to metabolism by glucocorticoids in chickens are considered to be homologous to mammals, although they lack the same information on a cellular level that mammalian research can provide. Numerous studies in poultry have demonstrated glucocorticoids can result in elevated blood glucose [22-26]. The source of increased glucose may either be associated with absorption in the intestine from exogenous sources, or may derive from upregulated gluconeogenesis processes in the liver [22, 26, 27]. The administration of dexamethasone has shown to reduce glucose transport through jejunal brush border membrane vesicles when administered for 7 days [23]. The influence of glucocorticoids on glucose absorption may be dependent on both the dose and duration of glucocorticoid exposure. Given the evidence in mammals which supports gluconeogenesis occurring in the presence of glucocorticoids, a similar process in poultry likely exists, however, an understanding of precise physiological mechanisms is limited.

1.2.2.3 Lipid metabolism in mammals

Hyperactive HPA activation leads to abnormal fat deposition and hepatic steatosis [16, 28]. In a broad context, glucocorticoids stimulate lipid synthesis (lipogenesis) in the liver, and decrease the mobilization of lipids, leading to triglyceride accumulation in hepatocytes [28]. Lipogenesis induced by glucocorticoids appears to require insulin signaling [16]. Glucocorticoids have been shown to regulate the expression of some genes involved in insulin-mediated lipogenesis [29].

As indicated above, glucocorticoids can drive the production of glucose, which provides a substrate for *de novo* lipogenesis [28]. Acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) are rate-determining enzymes involved in the initiation of lipogenesis. Glucocorticoids can control the expression of ACC and FAS, as glucocorticoid binding sites have been identified near promoter regions of these genes [16]. Several other lipogenic proteins have also been reported to be regulated by the glucocorticoids, including stearoyl-CoA desaturase, glycerol-3-phosphate acetyltransferase, 1-acylglycerol-3-phosphate acyltransferase, lipin-I, and diacylglyceride acyltransferase [16, 30]. The LXR is

also implicated in glucocorticoid-mediated lipid metabolism; in this regard, LXR knockout mice exhibited reduced accumulation of triglycerides when CORT was administered [31].

Glucocorticoids can also impact the mobilization of lipids, which is dependent on very-low density lipoprotein (VLDL) production [16]. Key components to VLDL production are apolipoprotein B (APOB) and microsomal triglyceride transferase protein (MTTP) [32]. Lipids bind to ApoB while MTTP catalyzes the assembly of VLDL [32]. Mice administered dexamethasone showed enhanced VLDL production and hypertriglyceridemia [33]. The enhancement of VLDL production may be due to glucocorticoids stabilizing the expression of APOB, and/or to the synergistic effect of increased lipid levels [16]. Alternatively, the mobilization of triglycerides from adipose tissue may contribute to the development of hypertriglyceridemia [28].

Disruption of negative regulatory pathways to control glucocorticoid levels have been implicated to promote hepatic steatosis. The enzymes, 11-beta-hydroxysteroid dehydrogenase and A-ring reductases, function to deactivate glucocorticoids [28]. Dysregulation of theses enzymes can facilitate the over activation of glucocorticoids, and this has been linked to the development of non-alcoholic fatty liver disease (NAFLD) and inflammatory metabolic diseases [34, 35]. Furthermore, early phase NAFLD with efficient clearance of glucocorticoids can protect against steatosis [25].

1.2.2.4 Lipid metabolism in poultry

Birds subjected to stress show increases in fat deposition in the abdomen, thigh muscle, and liver [22, 36, 37]. Increased circulating triglycerides and glucose, accompanied with increased insulin suggests that insulin resistance occurs under stress as in mammals [22, 38, 39]. The liver is the primary site for *de novo* lipogenesis in chickens where a conversion of energy into fat stores under stress has been shown in several studies [40]. Glucocorticoid administration in chickens results in increased expression of FAS, ACC, and ME, and additionally, increases activity of ME and FAS in the liver [36, 39, 41]. High levels of ammonia exposure have also resulted in increased expression of ACC and ME in the liver, which demonstrates the impacts that production stressors can have on bird metabolism [42]. Elevated fatty acid binding protein mRNA in the liver, along with APOC3, support upregulation of fat export from the liver [36, 43]. Other studies have suggested that the lack of APOB may result in lipid retention in the liver [39]. Nonetheless, increased lipid export from the liver corresponds to multiple studies that demonstrated increased triglycerides, VLDL, and cholesterol under glucocorticoid exposure [22, 24, 36, 39]. Increased cholesterol synthesis also reportedly occurs in birds subjected to stress [36]. The administration of CORT in drinking water resulted in increased liver [44] and breast and leg muscle cholesterol [45]. Increased mRNA of the rate controlling enzyme in cholesterol synthesis, HMGCR (3-

hydroxy-3-methyl-glutaryl-coenzyme A reductase), was increased in the breast muscle under CORT administration and supports stress-induced impacts on meat quality [45].

1.2.2.5 Protein metabolism in mammals

Excess glucocorticoid levels can decrease the size of muscle fibers and lead to muscle atrophy [17]. Glucocorticoids affect glycolytic-type muscle fibers, such as fast-twitch or type II fibers [46]. Elucidation of the precise mechanisms are still under investigation, but may be linked with higher levels of the glucocorticoid receptor in these muscle fibers [17, 46]. The activation of protein breakdown and decreased protein synthesis are two processes affected by synergistic actions of glucocorticoids in muscle tissue to induce atrophy [17].

(i) Activation of proteolytic systems. Glucocorticoids can activate proteolytic systems, primarily the ubiquitin proteasome system; although, lysosomal and calcium-dependent protein degradation are also thought to be mediated by glucocorticoids [47]. One way in which glucocorticoids mediate the activation of ubiquitin proteome systems is through the stimulation of myostatin, as its promoter region contains a GRE [48]. Myostatin is a growth factor that inhibits muscle mass production [17]. Myostatin increases the expression of the transcription factor, FOXO [49]. The overexpression of FOXO results in muscle atrophy, as it activates several atrogenes (genes involved in atrophy) [50] [51, 52]. More specifically, Atrogin-1 and MuRF-1 are muscle specific E3 ubiquitin ligases that can facilitate the ubiquitination of protein substrates [17]. Therefore, glucocorticoids indirectly stimulate the expression E3 ubiquitin ligases, Atrogin-1 and MuRF-1, through the promotion of myostatin signaling [17].

(ii) Reduction of protein synthesis. The suspension of protein synthesis also contributes to glucocorticoid-induced muscle atrophy. The interference with Insulin-like Growth Factor I (IGF-I) by glucocorticoids can result in both suppression of protein synthesis and increased proteolytic activity [53]. The IGF receptor signals through the PI3K-Akt pathway to mediate anabolic activity in muscle cells [17]. The serine-threonine kinase, mTOR, follows PI3K-Akt in the signaling pathway, and it is inhibited by glucocorticoids. This kinase is responsible for the phosphorylation of the proteins, 4E-BP1 and S6K1, which are mediators of protein synthesis [54]. Furthermore, glucocorticoids can stimulate the transcription of REDD1 and KLF-15, which suppress mTOR signaling and can prevent amino acid transport into cells [55]. Both KLF-15 and FOXO can act synergistically to promote the expression of atrogenes. Collectively, glucocorticoid interference in mTOR signaling results in decreased mRNA translation and decreased protein synthesis [17].

1.2.2.6 Protein metabolism in poultry

Production stressors (i.e. housing density, ventilation, heat stress) and glucocorticoid administration can impede muscle growth and promote muscle atrophy in chickens. As with mammals, evidence in chickens supports glucocorticoid-mediated activation of muscle atrophy and the reduction of protein synthesis. The metabolite, 3-methylhistidine, is a commonly used marker of protein breakdown in poultry as it is a non-metabolizable amino acid [56]. Corticosterone administration and heat stress in poultry have shown to increase 3-methylhistidine suggesting that stress can promote muscle atrophy and muscle wasting [27, 57]. The activation of the muscle specific E3 ubiquitin-ligase, atrogin-1, along with its transcription regulators, FOXO1 and FOXO3, have been examined in poultry. For example, a single dexamethasone treatment in chicks resulted in increased mRNA of atrogin-1, although mRNA expression of other proteolytic enzymes were unaltered [58]. Likewise, heat stress also induced elevated atrogin-1 mRNA, which also corresponded with elevated CORT; upon CORT returning to baseline, atrogin-1 no longer remained elevated [57]. This study showed a link between heat stress, CORT production, and the expression of antrogin-1 mRNA. Additionally, this study showed an increase in CORT and atrogin-1 after 12 hr of heat stress, which was associated with the elevation of 3-methylhistidine when heat stress persisted for 3 days [57]. As stated earlier, overexpression of transcriptional regulators, FOXO, can result in muscle atrophy. However, only modest increases in FOXO3 mRNA after 12 hr of heat stress was observed in chickens, and levels returned to baseline by 1 day after exposure [57].

It has been suggested that a decrease in ATP generation and metabolic intermediates during glucocorticoid exposure may inhibit the utilization of amino acid intermediates and reduce the capacity for protein synthesis in chickens [59]. The mTOR signalling pathway is a positive regulator for protein synthesis where decreased oxygen and ATP can result in decrease mTOR signalling [60]. The phosphorylation of mTOR leads to its activation, subsequent phosphorylation of S6K1, and increased transcription of processes involved in protein synthesis [60]. Administration of dexamethasone has been shown to inhibit the phosphorylation of mTOR and S6K1 in breast muscle suggesting that mTOR signalling is a pathway involved in glucocorticoid-induced muscle atrophy in chickens [61].

1.2.3 Stress and immune function

Information on how glucocorticoids influence immune responses in chickens is limited in comparison to mammals. The lack of mechanistic transgenic and knock-out poultry models and the inability to readily isolate and characterize specific cell types in chickens has impeded the understanding of how glucocorticoids impact immune responses in avian species. Furthermore, substantially more

knowledge gaps currently exist in the understanding of innate and acquired immunity of poultry in comparison to mammals. In general, avian immunology functions in a parallel manner to mammalian immunology with some notable differences. Avian species lack a lymphatic system with defined lymph nodes, and utilize a bursa of Fabricius, which is a diverticulum of the gastrointestinal tract responsible for the development of B cells [62]. To date, some metrics used to evaluate the impacts of glucocorticoids in poultry have included lymphoid organ size, immune indices (i.e. heterophil to lymphocyte ratio; H:L ratio), cytokine protein levels, cell death assays, and gene expression [63]. More recently, studies have utilized transcriptomics in an attempt to elucidate how glucocorticoids influence immune organs on a molecular level [64-66]. Presently, relatively few studies in poultry have provided information on specific immune cell types and trafficking.

1.2.3.1 Heterophil to lymphocyte ratio

Early research focused on characterizing the immunosuppressive function of glucocorticoids on poultry. This research guided the discovery and use of hallmark immune markers, such as the H:L ratio, that have been used for decades to evaluate immune function under stress. The H:L ratio has been shown to increase under stress due to the mobilization of immature heterophils from bone marrow into the periphery to aid in the initial threat response stimulated by stress [62, 67]. The decrease in peripheral lymphocytes is thought to be driven by the migration of lymphocytes to the site of infection/injury or lymphatic tissues [62]. Both the administration of glucocorticoids and exposures to production stressors have shown to increase the H:L ratio. Some studies have suggested that the H:L ratio is a more accurate measure of stress in an animal than measuring CORT concentrations [68, 69]. This is likely due to the delayed onset of H:L changes, and regulatory mechanisms involved in returning H:L to baseline levels when birds are exposed to a production stressor. Information obtained to date indicates that the release of glucocorticoids under a production stressor stimulates negative regulatory actions which can return CORT levels to baseline after an acute stressor [57, 70].

1.2.3.2 Impact of acute and chronic stress exposure on immune responses

Research in mammals has shown that glucocorticoids can result in a two-phased response on the immune system depending on the type, concentration, and duration of stressor [71]. For example, acute stress exposure can result in stimulatory actions to immune function, whereas chronic or repeated stress exposures result in immune suppressive activities [4]. Poultry research is in-line with these observations where stressors (e.g. production stressors or glucocorticoid treatment) applied for acute and chronic periods results in differing immune function [4].

(i) Acute Stress Impacts. 'Acute' stress is defined herein as exposure to a given stressor for 1 to 48 hr. Generally, glucocorticoids are considered immunosuppressive, and they have longed been used therapeutically for this purpose. However, acute exposure may result in permissive actions to the immune system, which derives from the host's need to be equipped to respond to a stressor or threat [71]. Mammalian research has suggested that stress influences on early immune responses result in increased inflammatory signalling [71]. In poultry, peripheral lymphocytes show increased expression of pro-inflammatory cytokines, *IL1B*, *IL6*, and *IL18* after 3 hr of CORT exposure in drinking water [72]. Interleukin-18 can be immunostimulatory during acute stress, and this cytokine is critical to initiating inflammatory responses and enhancing innate immunity [72]. Likewise, several chemokines (e.g. *CCLi2*, *CCL5*, *CCL16*, *CXCLi1*), exhibit increased expression in peripheral lymphocytes after 3 hr, and in splenocytes after 24 hr after exposure to CORT emphasizing the glucocorticoid-incited redistribution of immune cells in acute stress [72]. *CCLi2* is chemotactic to monocytes, lymphocytes, and macrophages, which may contribute to the redistribution of lymphocytes from blood to spleen [72]. A main attractant to heterophils in chickens is thought to be *CXCLi1*, and its upregulation is thought to aid in the recruitment of heterophils from bone marrow during glucocorticoid mediated stress [73]. Similar inflammatory expression patterns are observed in chicken heterophils, albeit in a more transient manner [74]. In this regard, at 3 hr post CORT exposure, *IL1B*, *IL6*, *IL10*, *IL12α*, *IL18* are increased in heterophils; after 24 hr exposure, inflammatory cytokines are downregulated [74].

(ii) Chronic Stress Impacts. Long-term stress exposure impacts avian immune function in a number of ways. Atrophy of lymphoid organs has been historically recognized as an indicator of persistent stress [75, 76]. The spleen, bursa, and thymus all demonstrate atrophy and morphological changes upon exposure to exogenous glucocorticoids or production stressors [36, 77, 78]. Heat stress results in fewer germinal centres in the spleen, which decrease B cell proliferation and the subsequent B cell-mediated immune response [77]. For example, heat stress has shown to decrease $Bu1^+$ B cells and $CD3^+$ T cells in the spleen [77]. Morphological changes to the bursa and thymus have also been observed [36, 77]. For instance, birds subjected to CORT administration showed lymphoid depletion and epithelial atrophy of the bursa along with increased expression of *IL6* and *TGFB* after 5 days of administration [36]. In the thymus, impaired maturation and selection of T cells was induced by heat stress [77]. Furthermore, ultrastructural changes included loss of structural borders and thymic cortex degradation with erythrocyte influx [77]. The destruction and atrophy to immune organs incited by stress undoubtedly hinders a bird's ability to mount an effective immune response. This supports much of the mammalian literature (and therapeutic use) of glucocorticoids as being immunosuppressive.

1.2.4 Stress and the intestine

1.2.4.1 Evidence from mammals

Glucocorticoids are either distributed to the intestine via the blood circulatory system, or can be produced locally by epithelial cells [79]. Pro-inflammatory cytokines and lipopolysaccharide are some stimuli that can promote the synthesis of glucocorticoids likely as a feedback mechanism to control inflammation [80, 81]. Glucocorticoids have an anti-inflammatory effect on immune and epithelial cells via activation of the glucocorticoid receptor as previously described [79]. Glucocorticoids may reduce certain immune cell types as restraint stress in mice has shown to reduce intraepithelial lymphocytes within the gut [82].

Various components of enteric mucosal barrier function are targets of glucocorticoid activities. The impact of stress on mucus production, tight junctions, IgA secretion, β-defensins, and Pattern Recognition Receptors (PRRs) have been previously explored [83]. The degree to which glucocorticoids impacts mucus secretion depends on the duration of the response. Acute stress exposure increased mucin release in the colon of rats [84, 85]. Conversely, in chronic stress situations, mucus depletion was observed [86]. The increase in mucin secretion in acute stress is thought to inhibit microbes from accessing the epithelium [87]. The advantage of mucus depletion in animals under chronic stress is less clear. The expression of MUC2 and MUC5AC have been shown to be downregulated after dexamethasone administration [88]. Mucins may be regulated in similar manner to inflammatory mediators, therefore, given that chronic glucocorticoid exposure downregulates inflammatory responses, it may also facilitate decreased mucus over time [89].

It is currently unclear whether glucocorticoids disrupt or enhance enterocyte tight junctions as reports are often contradictory. The effects of glucocorticoids on tight junction may be dependent on the cell type or species examined, or whether an inflammatory response is present. When human Caco-2 epithelial cells were exposed to dexamethasone, transepithelial electrical resistance (TEER) was increased in a dose and time dependent manner. Meanwhile, decreased expression of pore-forming protein, claudin (CLD) 2 was observed while increased expression of tight junction sealing protein, CLD4 occurred [90]. These effects were shown to be mediated by MAPK phosphatase; when this enzyme was inhibited and dexamethasone was administered, the alterations to TEER and CLD expression no longer occurred [90]. Glucocorticoids have been shown to enhance tight junctions in patients with inflammatory bowel disease, although, it is unknown whether this an indirect effect of anti-inflammatory responses or direct effect to tight junctions [91]. During an inflammatory response, TNF-α activated myosin light chain kinase (MLCK), which promoted contractions of peri-junctional acto-myosin

filaments and disruption of tight junction permeability [91]. The GC-GR complex interfered with TNF-α mediated production of MLCK by binding to a GRE in its promoter region [91]. Therefore, glucocorticoids can suppress tight junction permeability mediated by a TNF-α inflammatory response.

1.2.4.2 Evidence in poultry

Studies examining the impact of glucocorticoids on intestinal function in poultry are limited. Poultry subjected to heat stress resulted in decreased expression of *TLR2* in cecal tonsils [92]. This study also showed that heat stress decreased expression of avian β-defensins and sIgA in cecal tonsils, which was thought to contribute to increased *Salmonella enterica* colonization in birds receiving the heat stress treatment [92]. Heat stress in poultry has also been shown to increase the expression of *CLD5* and zona occluden 1 in the jejunum of broilers [93]. The administration of glucocorticoids in feed has shown to decrease small intestinal villus height and crypt depth [94]. The digestibility of dietary protein can also be reduced during glucocorticoid administration, along with an increased rate of epithelial cell proliferation in the small intestine [94]. Aside from glucocorticoids, catecholamine's, which are associated with stress, have been shown to increase intestinal transit time, which can facilitate the selection of fast-growing bacteria [79]. Catecholamine's may also directly interact with bacterial cell-surface receptors and modulate their virulence [95]. Collectively, more research in chickens is needed to facilitate a better understanding of how the mucosal environment and microbiota change under physiological stress mediated by glucocorticoids and if any interactions with other mediators, such as catecholamines are present.

1.3 Microbiota of poultry

1.3.1 Microbiota development in poultry

The importance of the intestinal microbiota for host health and poultry production has achieved widespread acceptance by the research community. Although mechanisms of action remain enigmatic, considerable effort is currently focused on linking the structure of the intestinal microbiota with production performance, which has prompted studies that examine the effects of microbial profiles on feed efficiency, immune system development, and food pathogen exclusion [96-98]. The elucidation of the poultry intestinal microbiota has previously been examined using culture-based methods and DNA fingerprinting methods; however, recent advancements in next-generation community sequencing targeting the 16S rRNA gene have supplanted other methods to characterize the structure of microbial communities [96, 99].

Chickens acquire their enteric microbiota in a unique manner relative to mammals; there is limited maternal transfer of microorganisms, eggs may be washed/sanitized, and the eggs are often hatched in

a relatively hygienic environment [5]. Production birds receive their initial exposure of microorganisms from their egg shell during hatching, and from the consumption of bedding, feed, water, and other environmental factors after hatching [5]. Differences between hatching environments and farms result in marked variations among the composition of bacterial communities in birds, and a defined 'core' poultry microbiota has yet to be identified [8, 100]. Some contributing factors to the large variations observed in structure of the enteric bacterial community in chickens has been attributed to bird age, breed, housing, diet, diet additives, maternal antibodies, and housing conditions [96, 100]. Furthermore, technical methods in the laboratory during sequencing analysis can also influence the identification of microbes in the intestine [100]. The majority of studies conducted to date have focused on characterizing the microbiota of ceca, with few studies examining the microbiota within small intestine [8]. Nonetheless, some studies have observed the small intestine to contain *Lactobacillus*, *Enterococcus*, *Clostridium sensu stricto*, and members of Clostridium XI cluster (genus *Romboutsia*, family Peptostreptococcaceae) [8, 101-103]. Although the distinction between allochthonous and autochthonous bacteria (e.g. mucosa-associated taxa) in the small intestine remains largely unexplored. In the ceca of Ross broiler chickens, the first few days after hatch results in the colonization of the phyla, *Firmicutes* and *Proteobacteria*, to dominate [100, 104]. After 3 weeks, bacteria in *Bacteroidetes* and *Firmicutes* become dominant and the microbiota begins to stabilize at this time [100]. The lack of microbial stabilization in young chicks potentially makes them vulnerability to disease development as their immune system is under developed, and a low diversity microbiota may enhance the ability of pathogens to colonize the intestine.

1.3.2 Colonization resistance

An important function of the intestinal microbiota is protecting the host from pathogen invasion [105]. This process has been termed 'colonization resistance' and is comprised of differing microbe-microbe and microbe-host interactions [106]. Stecher and Hardt [106] have proposed the following three primary mechanisms to define colonization resistance: (i) direct inhibition; (ii) nutrient competition; and (iii) stimulation of immune defenses [106]. Direct inhibition is a proposed microbe-microbe interaction that involves one bacterial species directly inhibiting the growth of another through the release of inhibitory compounds [106]. Nutrient competition is an interaction that involves differing bacterial species competing for similar nutrients and adhesion receptors under a reduced nutrient condition with the host [106]. In this regard, only one taxon can occupy a niche. The stimulation of immune defenses is a complex microbe-host mechanism that involves the microbiota inducing the ability of a host to mount an effective immune response, such as the release of various host-defense

peptides (e.g. defensins), mucins, and secretory IgA to protect against enteric pathogens [106]. Chicks develop diversity in their intestinal microbiota within the first week of life as they are exposed to new environmental constituents and begin to develop functional immunity. Importantly, delays in developing a complex microbiota may affect colonization resistance against pathogens and render them more susceptible to pathogenic infections early in the production cycle [6].

1.3.3 Stress and the microbiota

Increasing research is focusing on the bi-directional influence of the host-microbiota relationship as it relates to host health. Given the health misalignments with metabolism, immune function, and intestinal health under physiological stress, it is probable the microbiota is affected by stress. It is currently known that physiological stress alters intestinal morphology, increases digesta transit time, promotes mucus secretion, and alters intestinal permeability [107]. These processes can alter the nutrients available in the intestine and may promote a dysbiosis. Limited research has investigated the influence of physiological stress on the intestinal microbiota in poultry and its relationship to intestinal disease. Heat stress in poultry has been shown to alter the composition of bacteria in ileum and cecal digesta, and in feces [108-110]. Studies in mice have shown that social stress can decrease bacterial diversity and increase mucosal translocation of bacteria [111]. Furthermore, putatively beneficial bacteria, such as *Lactobacillus* spp., can decrease in abundance under conditions of host stress [107, 112]. It is possible that stress-incited impacts on the enteric microbiota of chickens, particular in young birds, may result in increased susceptibility to intestinal diseases. This warrants investigation. Importantly, achieving an understanding of the mechanisms involved is necessary to develop tailored and effective mitigations.

1.4 Necrotic enteritis

1.4.1 *Clostridium perfringens*

Clostridium perfringens is a gram positive, endospore-forming bacterium that has been recognized as a pathogen of human beings and livestock for over a century [95]. In the case of chickens, *C. perfringens* is the incitant of the economically-important disease, NE. The ban of many in-feed antibiotics has led to increased prevalence of NE in poultry flocks [113]. In most instances, *C. perfringens* is a commensal at low cell densities (10^2-10^4 CFU/g) [114]. This has stimulated interest in many researchers to determine what factors regulate the transformation of *C. perfringens* from a commensal to a pathogenic state. Several studies have focused on pathogenic strains of *C. perfringens* that possess putative virulence factors that are unique to specific genotypes of the bacterium. Traditionally, it was thought that chromosomally encoded alpha toxin (α-toxin) was responsible for the development of NE

within poultry [115]. More recently, a plasmid encoding toxin, NE β-like (NetB) toxin, has shown to be an important virulence factor [116]. Although the mechanisms of action of NetB toxin resulting in host cell necrosis are currently unresolved, the toxin thought to form a heptameric oligomer pore on the surface of host cells and thereby induce cell death [116].

1.4.2 Clinical pathology

Clostridium perfringens infections in production facilities often go unnoticed as it results in sudden death without conspicuous manifestation of pathognomonic signs or symptoms of disease [117]. Birds may present with behavioural changes such as resting its head down in addition to exhibiting other evidence of lethargy [117]. Gross pathological changes include swelling of the small intestine with a yellow pseudofibrinous membrane [114, 117]. Necrotic lesions are visible primarily within the jejunum, with some lesions occurring within the duodenum. It is thought that both NetB toxin and mucolytic enzymes released by *C. perfringens* are involved in lesion formation [114, 118]. A study that infiltrated crude toxins of *C. perfringens* directly into the duodenum of chickens showed marked edema and detachment of epithelium from lamina propria within 3 hr post exposure to the toxin [119, 120]. At 5 hr post exposure, necrosis of epithelium and lamina propria with infiltrations of mononuclear cells and heterophils were observed, and at 8-12 hr post exposure massive necrosis to villi and necrotic regions spanning to the bottom of the crypts occurred [120]. Necrosis to mucosal epithelium is not thought to occur due to direct damage. Instead, it has been described that mucosal necrosis arises from the necrotic death of enterocytes which occurs due to lamina propria destruction, and breakdown of both extra-cellular matrix and intercellular junctions [121].

Successful induction of lethal *C. perfringens* infection without a co-infection of coccidiosis agent has not been achieved [120]. There is a general consensus that disease induction due to *C. perfringens* alone in a laboratory setting results only in subclinical and variable clinical symptoms of disease [114]. Furthermore, genetics of the bird influence disease severity; broilers may be more susceptible to NE than layer breeds [115]. The subclinical form of NE presents with chronic mucosal damage, leading to a decrease digestion and absorption, ultimately resulting in negative performance and inefficient food conversion rates [122].

1.4.3 Pathogenesis and predisposing factors

The mechanisms of *C. perfringens* pathogenesis in chickens remains enigmatic. Several studies focus on the toxins released by *C. perfringens* which are undoubtedly a critical role in the facilitation of necrotic tissue damage [114]. Gaining close proximity to a host's mucosa is an important strategy for most pathogens. Many enteric pathogens use motility strategies, such as a flagella or pilli, to gain access

to the mucosa [123]. *Clostridium perfringens,* however, does not possess either of these motility strategies to penetrate mucus. Emerging evidence indicates that the ability of the bacterium to degrade mucus may be important in pathogenicity [124]. *Clostridium perfringens* has been shown to utilize mucins as a sole source of energy, suggesting that *C. perfringens* may colonize the mucus layer of the intestine [124]. Furthermore, research has demonstrated that the NetB-containing *C. perfringens* strain, CP1, is capable of modifying the glycan component of chicken intestinal mucin supporting its potential to traverse the mucus layer in the intestine as a first step in gaining access to the host epithelium [125].

As indicated previously, *C. perfringens* is non-pathogenic at lower cell densities (10^3-10^4 CFU/g) and is a pathogenic at higher cell densities (10^8-10^9 CFU/g) [114]. This is consistent with the possibility that an environment change within the intestine facilitates proliferation of *C. perfringens* allowing pathogenesis [114]. Several factors have been proposed to explain the environmental perturbations including mucosal damage, or modulation of mucus production and secretion [114, 115, 124]. Co-infection with a coccidiosis agent is thought to be necessary for *C. perfringens* to incite NE [114], and it is currently thought that the coccidiosis agent (e.g. *Eimeria maxima*) causes mucosal damage and facilitates *C. perfringens* movement towards the epithelium [114, 115, 124]. It has also been suggested that coccidian-induced tissue damage results in the release of plasma protein into the luminal region, which then promotes the proliferation of *C. perfringens* [126]. This protein source may be important for *C. perfringens* pathogenesis as the bacterium lacks the ability to synthesize many amino acids [126]. It is also thought that dietary factors play a critical role in the modulation of intestinal environment [114]. Several studies have indicated that the inclusion of non-starch polysaccharides and high protein content diet can result in increased mucin production, which may be associated with outbreaks of NE [114]. Provided that *C. perfringens* has mucolytic potential and it can sustain itself on mucus glycans as a sole energy source, it is conceivable that increased mucus production could be the link to inducing pathogenicity of *C. perfringens* [124].

Quorum sensing is a common strategy that numerous pathogens rely on to colonize and persist in microbiologically-complex habitats. In this regard, quorum sensing is a communication strategy that many microbes utilize to coordinate pathogenesis only under the circumstances of a high cell density in order to preserve energy [127]. Intra-species quorum sensing involves the release of a molecule from one bacterium into the extracellular space that can be sensed by surrounding microbes of the same species and elicit the coordinated expression of virulence factors, such as toxins [127]. It is known that release of the α-toxin in *C. perfringens* is under control of the VirR/VirS system; it may be that the NetB toxin and other virulence factors are also under the control of this system [128]. Recently it was shown

that the pathogenicity of *C. perfringens* strain CP1 required an Agr-like quorum sensing system, and that the VirR/VirS system was involved in the transcription of the NetB toxin gene [128, 129].

1.4.4 *Clostridium perfringens* and stress

Mounting pressure to eliminate the use of in-feed antibiotics in chicken production has prompted investigation into non-antibiotic measures to prevent diseases like NE. Even with the most vigilant management practises, birds in production are subjected to numerous stressors, which requires adaptation on a physiological level. There is a strong body of literature that focuses on exogenous predisposing factors (i.e. coccidia, diet, etc.) to NE with little consideration of factors that may vary within a host. Stress hormone, epinephrine, has been previously linked to *C. perfringens* through observations in human medicine. For example, in the 1920's it was observed that syringes contaminated with endospores of *C. perfringens* were associated with occurrence of gas gangrene infections in patients receiving epinephrine treatment [95]. Furthermore, stress research in mice has shown that social stressors decrease densities of bacteria within the genus *Bacteroides* while increasing the abundance of bacteria within the genus *Clostridium* [111]. The association between stress and *C. perfringens* is beginning to be considered among poultry researchers. Heat stress, chilling, and social stress have all been demonstrated to increase densities of *C. perfringens* in the ceca [130-132]. These observations support stress as a potential predisposing factor to NE. However, stress, may not always present in a negative manner in the context of disease. For example, heat stress in poultry has shown to decrease macroscopic lesions in birds challenged with *C. perfringens* [133]. It may be that combined stressors, or repeated stressors, are more significant in promoting disease, as continual challenges to immune function, barrier function, metabolism, and microbiota will disrupt homeostatic functions. Thus, a major objective of my thesis research is to examine the interplay between stress impacts on chickens and the proliferation of *C. perfringens* in the intestine leading to the manifestation of disease. My studies utlilize the stress hormone, corticosterone, to mediate a stress response and attempt to replicate stress levels produced in a production setting.

1.5 Microbiota transplant as an antimicrobial alternative in poultry

Microbiota transplantation refers to the transfer of a community of microorganisms from one individual (i.e. healthy) to another individual (i.e. usually diseased or has pathologies) [9]. Traditionally, microbiota transfers include all microorganisms, such as bacteria, virus, fungi, archaea, and protozoa [134]. Microbiota transplant has been attempted in poultry to examine the role of the microbiota in feed efficiency and behavioural habits. Two studies conducted to date examined whether taking cecal or fecal contents from high performing chickens and administering the substrates to chicks via egg or oral

administration could transfer feed conversion qualities [135, 136]. Both studies concluded that high bird performance could not be transferred through the microbiota, and that other host and environmental factors were more involved in determining bird performance [135, 136]. The transfer of behavioural qualities through microbiota transplant, such as feather pecking, has also been examined [137]. Birds receiving a microbiota from high feather pecking donors showed increased activity responses (i.e. vocalization to stimulus); however, feather pecking behaviour was not transferred [137]. An obstacle in microbiota transfers is applying it in a manner that retains taxa that are obligate anaerobes and not predominated by endospore-forming bacteria. A recent study showed that the application of cecal contents to eggs primarily transferred endospore-forming bacterial taxa, including bacteria within the *Lachnospiraceae* and *Ruminococcaceae*, but it was not successful at transferring bacteria belonging to other important taxonomic groups, such as *Bacteroidaceae* and *Lactobacillaceae* [138]. Additional research is needed to overcome this obstacle and achieve colonization of the gastrointestinal tract by diverse obligate anaerobic bacteria. The application of microbiota transplantation in preventing or treating disease in poultry is yet to be considered. An early publication in poultry has shown that fecal transplants from adult birds to broiler chicks could reduce the number of *Salmonella* carriers in the ceca [139]. Although microbiota transplants to prevent or treat disease have been done in other species, such as swine, few papers have examined the mechanisms involved [9]. A potential issue with the administration of digesta or feces is the inadvertent transfer of pathogens, and researchers advancing the use of fecal transplants in human beings are attempting to address this issue by developing community probiotics [9]. In addition to ascertaining efficacy of microbiota transplantation, the elucidation of mechanisms involved is crucial to optimize the advancement of this strategy as an alternative to antibiotics in poultry (i.e. mechanisms of colonization resistance, identification of bacteria conferring colonization resistance).

1.6 Hypotheses and objectives

1.6.1 Objective 1 – Evaluate the impact of physiological stress on poultry health

Hypothesis: The administration of CORT to chickens will result in modulations to host metabolism, systemic responses, and intestinal microbiota.

Research addressing this objective is presented in Chapters 2 and 3. Birds were administered CORT in their drinking water to mediate a stress response that promotes HPA axis and mimicks the physiological impacts that production stressors can elicit. Lipid metabolism was examined through histopathology and mRNA gene expression. Metabolomics was conducted on liver, kidney, and breast muscle tissues to identify metabolic changes occurring that were mediated by CORT. The bursa of

Fabricius was examined through histopathology and mRNA gene expression to determine if CORT promoted immunosuppressive functions. Lastly, the intestinal microbiota of the small intestine and ceca was examined through 16S rRNA gene sequencing to evaluate how diversity and microbial communities are altered by stress.

1.6.2 Objective 2 — Examine the impact of stress on the pathogenesis of *Clostridium perfringens*

Hypothesis: Co-challenging birds with CORT and *C. perfringens* will increase densities of *C. perfringens* in the small intestine, induce subclinical NE, and result in reduced weight gain in layer breed chickens.

Research addressing this objective is presented in Chapter 4. Layer birds were co-challenged with CORT in their drinking water and orally inoculated with *C. perfringens*. Densities of *C. perfringens* were examined to test the hypothesis in question. Histopathology was completed in the small intestine to evaluate disease progression. Several genes were examined through mRNA gene expression in the intestine, spleen, and thymus to examine mucus, barrier function, and immune responses. Weight gain was measured throughout the study to ascertain how co-challenging birds with CORT and *C. perfringens* impacts bird performance.

1.6.3 Objective 3 — Development of a broiler chicken NE model using inoculation of virulent *Clostridium perfringens* and in-feed corticosterone administration

Hypothesis: Challenging broiler birds with *C. perfringens* and CORT will induce NE and produce greater incidence of clinical pathologies in comparison to layer model.

Research addressing this objective is presented in Chapter 5. Broiler birds were co-challenged with CORT and *C. perfringens* to induce NE. Metric of disease were evaluated. These included measuring densities of *C. perfringens*, gross and histopathological examination, and quantifying mRNA gene expression of inflammatory markers in the small intestine.

1.6.4 Objective 4 — Administer a complex microbiota to expedite the establishment of a core microbiota in day-old chicks as a method to mitigate stress-induced disease

Hypothesis: The introduction of a complex microbiota to day-old broiler chicks will provide colonization resistance when exposed to stress and *C. perfringens*.

Research addressing this objective is presented in Chapter 6. A complex microbiota was produced through propagating cecal contents obtained adult broiler breeders in a anaerobic bioreactor system. Day-old chicks were administered the complex microbiota and then subsequently challenged to induce NE after 10-days-of-age. Birds were co-challenged with CORT and *C. perfringens* to induce NE. The intestinal microbiota was examined through 16S rRNA gene sequencing and NMR-based metabolomics to determine how microbial communities and metabolites may function in disease resistance. Disease

progression was evaluated through gross and histopathological evaluation and quantifying *C. perfringens in* the small intestine. Several genes were examined in the small intestine to determine how administering a complex microbiota can influence mucus, barrier function, antimicrobial, and immune responses during a pathogen challenge.

Chapter 2: Corticosterone-mediated physiological stress modulates hepatic lipid metabolism, metabolite profiles, and systemic responses in chickens[1]

2.1 Abstract

The impact of physiological stress on lipid metabolism, the metabolome, and systemic responses was examined in chickens. To incite a stress response, birds were continuously administered corticosterone (CORT) in their drinking water at three doses (0 mg/L, 10 mg/L, and 30 mg/L), and they were sampled 1, 5, and 12 days after commencement of CORT administration. Corticosterone administration to birds differentially regulated lipogenesis genes (*i.e. FAS*, *ACC*, *ME*, and *SREBF1*), and histopathological examination indicated lipid deposition in hepatocytes. In addition, CORT affected water-soluble metabolite profiles in the liver, as well as in kidney tissue and breast muscle; thirteen unique metabolites were distinguished in CORT-treated birds and was consistent with the dysregulation of lipid metabolism due to physiological stress. Acute phase responses (APRs) were also altered by CORT, and in particular, expression of *SAA1* was decreased and expression of *CP* was increased. Furthermore, CORT administration caused lymphoid depletion in the bursa of Fabricius and elevated *IL6* and *TGFβ2* mRNA expression after 5 and 12 days of CORT administration. Collectively, incitement of physiological stress via administration of CORT in chickens modulated the host metabolism and systemic responses, which indicated that energy potentials are diverted from muscle anabolism during periods of stress.

2.2 Introduction

Poultry are subjected to numerous stressors throughout their lifetime. Temperature changes, social stress, and transportation are some consequences of the production cycle that can elicit a stress response in birds [140, 141]. It has been demonstrated that physiological stress can impact production performance by altering various host functions. Meat quality, weight gain, and feed efficiency have been shown to be modulated by stress and are some examples of how stress can negatively impact production performance [3, 26, 27].

Previous studies in poultry have demonstrated that physiological stress can result in increased expression of hepatic lipid synthesis genes and extrahepatic lipid deposition [26, 41]. *De novo* lipogenesis primarily occurs in the liver and to a lesser extent in adipose tissue of poultry [142]. Fatty acid synthase (*FAS*) is a multifunctional enzyme that uses NADPH to catalyze malonyl-CoA into palmitate

[1] A version of this chapter has been previously published: Zaytsoff, S.J.M., Brown, C.L.J., Montina, T., Metz G.A.S, Abbott W.A., Uwiera R.R.E., Inglis G.D. Corticosterone-mediated physiological stress modulates hepatic lipid metabolism, metabolite profiles, and systemic responses in chickens. *Sci Rep* **9,** 19225 (2019).

[143]. Malonyl-CoA is generated from the carboxylation of acetyl-CoA by acetyl-CoA carboxylase (*ACC*); additionally, this enzyme acts as the rate limiting step in fatty acid synthesis [40]. The hydrogen donor, NADPH, in *de novo* lipogenesis derives from the catalysis of malate by malic enzyme (*ME*) or from glucose catabolism in the pentose phosphate pathway [143]. Lipogenic gene expression is mediated by sterol regulatory element binding transcription factor (*SREBF1*) and liver nuclear X receptors [40]. Elevated levels of *SREBF1* corresponded with increased expression of cholesterol and fatty acid synthesis genes [40]. Synthesized lipids are then incorporated into very-low density lipoprotein (VLDL) for secretion from the liver where they enter circulation for deposition into extrahepatic and adipose tissues [144].

The use of metabolomics in poultry research has increased in recent years and is currently being used to better understand a variety of host responses [145, 146]. Examining the metabolome of tissues, blood, urine, and feces has created a novel method to assess the regulation of metabolic pathways. Furthermore, defined metabolite signatures that occur during disease can be used to develop novel biomarkers. Corticosterone (CORT) is a glucocorticoid released upon stimulation of the hypothalamic-pituitary-adrenal axis in chickens and results in varying physiological changes needed to regain homeostasis [147]. Corticosterone treatment in broiler chickens has been shown to increase plasma levels of amino acids, suggesting modulations to protein metabolism [27]. Elucidating modulations to metabolism under physiological stress through the use of metabolomics may aid in the development of evidence-based and novel interventions. Such interventions could include feed additives or supplements that can be administered to birds under periods of stress and assist in restoring metabolic homeostasis.

Studies in cattle and swine have demonstrated an acute phase response (APR) can be elicited during physiological stress [148, 149]. An APR involves the synthesis of acute phase proteins (APPs) in the liver that results in a systemic response to early inflammation [63, 150]. The function of APPs in avian species is less defined than in mammals, although APPs in birds generally aid the host in restoring homeostasis and limiting microbial infection [151]. Ceruloplasmin (*CP*), transferrin (*TF*), and serum amyloid A1 (*SAA1*) are considered to be positive APPs in chickens with their expression increasing during an APR [151]. Zulkifli *et al* demonstrated both *CP* and *TF* to be transiently elevated after CORT administration in chickens [152]. A stress-induced APR and changes to immune status are expected to be metabolically costly, and may be responsible for modifications to metabolic metrics observed during physiological stress in chickens. The majority of research elucidating the impact of glucocorticoids such as CORTs on immune function suggests that they act as anti-inflammatory agents and suppress adaptive immune responses [71].

Understanding how metabolism and systemic responses are altered during physiological stress is essential to identifying mechanisms of reduced weight gain performance. I hypothesize that physiological stress will simultaneously modulate (i) hepatic lipid metabolism, (ii) the metabolome of breast muscle/kidney/liver tissues, and (iii) systemic physiological responses (i.e. acute phase and immune responses). To test these hypotheses, I administered CORT at two doses (10 mg/L and 30 mg/L) to birds through their drinking water as a method to simulate a stress response, and measured an array of host responses after 1, 5, and 12 days of CORT treatment. Histopathological changes and mRNA gene expression of lipid metabolism genes were measured in the liver as it is the primary site of lipid synthesis in birds [40]. Breast muscle, kidney, and liver tissues were subjected to metabolomic analysis to provide insights on how stress can modulate metabolite profiles and lead to biomarker discovery. The APR was examined through mRNA gene expression in the liver as this is the location where APP are synthesized [150]. Lastly, mRNA gene expression of immune genes and histopathology was examined in the bursa of Fabricius as atrophy has been previously reported to occur as the result of CORT administration [153].

2.3 Methods

2.3.1 Experimental design

The experiment was designed as a factorial experiment with four levels of stress treatment and three levels of time arranged as a completely randomized design. The four stress treatments were: CON (untreated drinking water); ECC (0.2% ethanol drinking water); LD-C (10 mg CORT per 1 L of drinking water); and HD-C (30 mg CORT per 1 L of drinking water). Birds were euthanized and sampled from each stress treatment at 1, 5, and 12 days (n=3 per treatment, n=36 total). Each replicate included 12 chicks, and were conducted on three separate occasions to ensure independence.

2.3.2 Ethics statement

The study was carried out in strict accordance with the recommendations specified in the Canadian Council on Animal Care Guidelines. The project was reviewed and approved by the Lethbridge Research and Development Centre (LeRDC) Animal Care Committee (Animal Use Protocol Review #1526) before commencement of the research.

2.3.3 Birds

Thirty-six specific-pathogen-free white leghorn chickens were used in this study. Eggs were purchased from the Canadian Food Inspection Agency (Ottawa, Canada). Eggs were incubated in a Brinsea Octagon 40 Advanced Digital Egg Incubator according to the manufacturer's guidelines for incubating chicken eggs (Brinsea Products Inc., Titusville, FL). Eggs were maintained at 37.5°C and 45%

humidity with hourly turning of the eggs for the first 18 days of incubation. Thereafter, eggs were set flat for hatching and humidity was increased to 60%. All hatched chicks were acclimatized in a group within one large animal pen (1.1 m^2) for 10 days and had access to a brooder (Brinsea Products Inc., Titusville, FL). Birds had *ad libitum* access to a non-medicated starter diet (Hi-Pro Feeds, Lethbridge, AB) and water at all times. Birds were maintained on a 12 hr light: 12 hr dark cycle. At 11 days-of-age, birds were randomly assigned to the four stress treatments and housed in groups of four within an individually ventilated cage (IVC) (Techniplast, Montreal, QC). Each animal cage contained a companion bird to ensure no birds were socially isolated. Birds were weighed daily throughout the study.

2.3.4 Corticosterone administration

Corticosterone treatment occurred as previously described [154]. Birds began receiving CORT when chicks reached 14-days-of-age and continued until the end of the experiment. Corticosterone (Sigma Aldrich Inc.) was dissolved in 2.0 mL of anhydrous ethanol and added to 1 L of drinking water. Water containing CORT was prepared fresh each day, and added to animal cages twice daily.

2.3.5 Animal euthanasia and sample collection

One bird per stress treatment was randomly sampled on day 1, 5, or 12 after initiation of CORT treatment. Birds were anaesthetized with isoflurane (5% isoflurane; 1 L O_2/min), and blood was collected by intracardiac puncture. Birds were then euthanized by cervical dislocation under general anaesthesia. The abdomen was opened with a ventral midline incision, and the viscera was examined grossly for changes in morphology. The liver, kidney, breast muscle, jejunum, and bursa of Fabricius were aseptically removed, and the bursa of Fabricius was weighed. Samples for RNA analysis were immediately placed within RNAlater® (Qiagen Inc., Toronto, ON). Tissues for histopathology were placed in 10% neutral buffered formalin. All remaining samples were stored at -80°C until processing.

2.3.6 Serum analysis

Concentrations of creatinine and cholesterol were measured using VetTest Chemistry Analyzer (Idexx Laboratories, Westbrook, ME). Dry slide technology panels specific to creatinine and cholesterol were used and manufactures guideline were followed for metabolite quantification.

2.3.7 Histopathology

Liver and bursa tissue samples were fixed in 10% neutral buffered formalin for minimum 24 hr. Samples were then dehydrated and embedded in paraffin blocks. Tissues were then sectioned (5 µm) using a microtome (Thermo Scientific™, Cheshire, UK), de-paraffinized with xylene, and stained with hematoxylin and eosin. Tissue sections were scored by a veterinary pathologist (R.R.E.U) blinded to treatments using modified scoring criteria previously described [155, 156]. Three tissue sections for each

animal and tissue type were examined to ensure uniformity of observations. Representative tissue sections were scored from a single field of observation within each histological section. Liver tissues were graded 0 to 4 for hepatocellular ballooning and fibrosis; 0 to 3 for macrovesicular steatosis, intra-acinar inflammation, portal tract inflammation, and Mallory's hyaline. Bursa tissues were graded 0 to 4 for lymphoid depletion, epithelial atrophy, hemorrhage/congestion, inflammation, fibrosis, and follicular cysts. Total pathological score was determined by calculating the sum of scores from all tissue assessment criteria for each bird.

2.3.8 Quantification of mRNA gene expression

Procedures for RNA extraction, reverse transcription, and quantitative PCR were followed as previously described [157]. Total RNA was extracted according to manufactures instructions from liver, bursa of Fabricius, and distal jejunum tissue using RNeasy mini kit (Qiagen Inc.). An additional DNase treatment (Qiagen Inc.) to extraction column was included to remove residual genomic DNA. A Bioanalyzer RNA 6000 Nano kit (Agilent, Mississauga, ON, Canada) was used to measure RNA integrity and quantity; and 1.0 µg of RNA was reverse transcribed to cDNA using QuantiTect reverse transcription kit (Qiagen Inc.). Reactions were run on a 384-well plate where each reaction contained 5.0 µL QuantiTect SYBR Green Master Mix (Qiagen Inc.), 0.5 µL of each primer (10 µM), 3.0 µL RNase-free water, and 1.0 µL cDNA. Quantitative PCR was performed using an ABI7900HT thermocycler (Applied Biosystems, Carlsbad, CA) with the following PCR conditions: 95°C for 15 min; 40 cycles of 95°C for 15 sec, 58-60°C for 30 sec, and 72°C for 30 sec; and melt curve analysis from 55-95°C. Primer sequences specific to gene targets (Appendix A, Table S1.1) were generated using NCBI primerBLAST; primers were designed to create an amplicon between 75 and 200 base pairs. Efficiencies for all primers were between 95-110% and a single peak was present in melt analysis. Reactions were run in triplicate and the average cT values were used to calculate gene expression relative to two reference genes (*Ba* and *Tbp)* using geNorm and qBase+ software (Biogazelle, Gent, Belgium) quantification model [158].

2.3.9 Tissue metabolomics

Tissue samples were processed as outlined in Wu *et al* [159] with the following modifications. Liver, breast muscle, and kidney tissue was homogenized in 4.0 mL/g methanol and 1.6 mL/g deionized water. Tissues were homogenized with 6-mm-diameter steel bead for 5 min intervals using Qiagen Tissue Lyser at 50 Hz followed by 1 min of vortexing. This step was repeated two additional times to ensure complete tissue homogenization. To each sample, 2.0 mL/g chloroform was added and vortexed thoroughly. Next, 2.0 mL/g chloroform and 4.0 mL/g deionized water was added to each sample and vortexed until thoroughly mixed. Samples were then incubated at 4°C for 15 min followed by centrifuging at 1000 x g

for 15 min at 4°C. Next, 700 µl of the supernatant was placed into a microfuge tube and left for 3-4 days to air dry. Samples were rehydrated in 480 µl metabolomics buffer (0.125 M KH_2PO_4, 0.5 M K_2HPO_4, 0.00375 M NaN_3, and 0.375 M KF; pH 7.4). A 120 µl aliquot of deuterium oxide containing 0.05% v/v trimethylsilylpropanoic acid (TMSP) was added to each sample (final total volume of 600 µl); TMPS was used as a chemical shift reference for ^{1}H-NMR spectroscopy. A 550 µl aliquot was then loaded into a 5 mm NMR tube and run on a 700 MHz Bruker Avance III HD spectrometer (Bruker, ON, Canada) for spectral collection. Data acquisition and processing were followed as previously described [160]. MATLAB (Math Works, MA, USA) was used for spectral peak alignment and binning using Recursive Segment Wise Peak Alignment [161] and Dynamic Adaptive Binning [162], respectively. The dataset was then normalized to the total metabolome, excluding the region containing the water peak, and pareto scaled. Metaboanalyst [163] was utilized for both the Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) model and the calculation of fold changes in specific metabolites. Metabolites were identified using Chenomx 8.2 NMR Suite (Chenomx Inc., AB, Canada).

2.3.10 Statistical analysis

Statistical analyses for gene expression, histopathology tissue scores, and weight gain were performed using Statistical Analysis Software (SAS Institute Inc. Cary, NC). Main effects within the experimental design were stress (n=4) and time (n=3) arranged as a completely randomized design. Gene expression data was assessed for normality and analyzed by a mixed linear model using the MIXED procedure of SAS. Body weight measurements were treated as a repeated measure; the appropriate covariance structure was utilized according to the lowest Akaike's Information criterion. In the event of a main treatment effect ($P \leq 0.050$), the least squares means test was used to compare treatments within factors for body weight and gene expression analysis. Histopathological data (i.e. categorical data) was analyzed using a non-parametric Fisher's exact test within SAS; pairwise comparisons was performed between all stress treatments at the 1, 5, and 12 day time points. Metabolomics analysis was performed using MATLAB (Math Works, MA, USA). Spectral bins were subjected to both univariate and multivariate analysis to determine which metabolites were significantly altered. The univariate measures were calculated using a decision tree algorithm as described by Goodpaster *et al* [164]. The multivariate tests utilized the Variable Importance Analysis based on random Variable Combination (VIAVC) algorithm, which combines both Partial Least Squares Discriminant Analysis (PLS-DA) and the area under the Receiver Operating Characteristics (ROC) curve to synergistically determine the best subset of metabolites for group classifications [165]. All P-values obtained from analysis were Bonferroni-Holm corrected for multiple comparisons. Orthogonal Partial Least Square Discriminate

Analysis (OPLS-DA) was carried out using the bins identified as significant by univariate and/or multivariate testing in order to observe group separation. Data is represented by mean ± standard error of the mean (SEM). Significance is indicated as * $P < 0.050$, ** $P < 0.010$, *** $P < 0.001$, and **** $P < 0.0001$.

2.4 Results

2.4.1 Corticosterone administration reduced weight gain efficiency

Weight gain was reduced ($P < 0.001$) for the 10 mg/L low dose CORT (LD-C) treatment in comparison to both the non-CORT control (CON) and ethanol carrier control (ECC) treatments (Figure 2.1). Weight gain was also reduced for the 30 mg/L high dose CORT (HD-C) treatment in comparison to the CON ($P < 0.001$) and ECC ($P = 0.006$) treatments.

2.4.2 Stress alters host physiology and hepatic histopathology

Gross morphological changes of the liver showed consistent enlargement and discolouration for the HD-C treatment 12 days post-CORT administration (Figure 2.2A). Total histopathological scores of birds receiving HD-C were consistently increased after 1 day ($P < 0.001$), 5 days ($P < 0.001$), and 12 days ($P < 0.001$) of CORT treatment in comparison to both the CON and ECC treatments (Figure 2.2C-E). Only birds receiving the LD-C treatment for 12 days had elevated total histopathological scores in comparison to CON treatment birds ($P = 0.031$; Figure 2.2E). Hepatocellular ballooning was a primary contributor to the elevated total histopathological scores observed for CORT treatment birds. The LD-C treated birds only demonstrated increased hepatocellular ballooning ($P \leq 0.025$) after 12 days of CORT administration where as the HD-C treated birds showed elevated hepatocellular ballooning after 1 ($P \leq 0.001$), 5 ($P \leq 0.001$), and 12 ($P < 0.001$) days of CORT treatment in comparison to the CON and ECC treatments. The HD-C treatment also resulted in increased macrovesicular steatosis after 5 days ($P < 0.023$) of CORT treatment and elevated Mallory hyaline after 5 ($P = 0.003$) and 12 ($P = 0.023$) days of CORT treatment. Serum cholesterol increased after 5 and 12 days of CORT treatment in birds for both the LD-C ($P \leq 0.038$) and HD-C ($P \leq 0.010$) treatments (Figure 2.2F).

2.4.3 Corticosterone modulates mRNA gene expression of hepatic lipid synthesis and secretion genes

Birds receiving LD-C or HD-C demonstrated up-regulation of FAS 1 day ($P = 0.045$), 5 days ($P = 0.038$), and 12 days ($P = 0.021$) post-CORT administration relative to CON treatment birds (Figure 2.3A). These results are consistent with the elevated *ACC* expression that was observed in birds administered CORT for 1 day at the high dose ($P \leq 0.002$). Only birds administered CORT at the low dose demonstrated elevated *ACC* expression after 12 days of CORT administration ($P \leq 0.003$). The HD-C treatment showed decreased *ACC* expression ($P \leq 0.013$) after 5 and 12 days of CORT administration

relative to expression levels after 1 day of CORT (Figure 2.3B). The expression of *ME* was increased ($P \leq 0.023$) after 12 days of CORT administration for both the LD-C and HD-C treatments (Figure 2.3C). Increased expression of *SREBF1* was observed in birds receiving LD-C for 12 days ($P \leq 0.011$), which supports upregulation of lipid synthesis (Figure 2.3D). The expression of *SREBF1* was elevated ($P \leq 0.022$) in HD-C-treated birds after 1 day of administration, but as observed with *ACC* expression, showed downregulation ($P = 0.003$) after 12 days of CORT treatment (Figure 2.3D). No changes in expression ($P > 0.100$) were observed for apolipoprotein B (*APOB*; Figure 2.3E); however, increased ($P \leq 0.026$) expression of apolipoprotein C3 (*APOC3*) was observed in birds receiving CORT after 1 and 12 days of administration (Figure 2.3F). The expression of microsomal triglyceride transferase protein (*MTTP*) was increased ($P \leq 0.050$) at all time points in birds administered CORT at a low dose in comparison to CON treatment. Birds administered CORT at a high dose only demonstrated elevated *MTTP* expression ($P \leq 0.026$) at the 1 day post-administration time point (Figure 2.3G).

2.4.4 Corticosterone treatment alters metabolite profiles

Water-soluble metabolites were extracted from liver, breast muscle, and kidney tissues and analyzed by ^{1}H-Nuclear Magnetic Resonance (NMR) spectroscopy to investigate metabolome changes associated with CORT administration. Analysis of both treatment groups included 439, 460, and 379 metabolite bins from the kidney, liver, and breast muscle, respectively. The LD-C and HD-C treatments were compared to ECC treatment birds to eliminate metabolite bias as a result of the ethanol carrier. Supervised OPLS-DA separation indicated that CORT administered at the low and high doses affected metabolite profiles in kidney (LD-C: $P = 0.001$, $Q^2 = 0.709$, $R^2 = 0.888$; HD-C: $P = 0.001$, $Q^2 = 0.888$, $R^2 = 0.960$), liver (LD-C: $P = 0.004$, $Q^2 = 0.846$, $R^2 = 0.995$; HD-C: $P = 0.001$, $Q^2 = 0.804$, $R^2 = 0.939$), and breast muscle (LD-C: $P = 0.001$, $Q^2 = 0.665$, $R^2 = 0.931$; HD-C: $P = 0.001$, $Q^2 = 0.904$, $R^2 = 0.938$) (Figure 2.4A-F). Corticosterone administration significantly affected quantities of several metabolites in tissues (Figure 2.5). In this regard, CORT treatment was associated with an increase ($P \leq 0.032$) in lactate and alanine, and a decrease ($P \leq 0.009$) in 3-hydroxybutyrate in the liver. Kidney tissue showed that CORT administration resulted in an increase ($P \leq 0.031$) in choline and creatinine, and a decrease ($P \leq 0.31$) in lactate. In breast muscle, CORT administration was associated with an increase ($P \leq 0.018$) in fucose, glucose, betaine, choline, taurine, and creatinine, and a decrease ($P \leq 0.035$) in β-alanine, aspartate, carnosine, and anserine. Serum creatinine levels were elevated in CORT treatment birds ($P \leq 0.050$).

2.4.5 High dose corticosterone treatment is associated with histopathological changes in the bursa of Fabricius

Total histopathological scores of bursa of Fabricius tissues were higher in HD-C treatment birds at 1 ($P < 0.001$), 5 ($P < 0.001$), and 12 ($P < 0.001$) days relative to ECC and CON treatment birds (Figure 2.6A-C). Elevated scores in HD-C treatment birds were attributed to higher lymphoid depletion (day 1, $P < 0.001$; day 5, $P \leq 0.035$); day 12, $P \leq 0.041$), epithelial atrophy (day 5, $P \leq 0.023$; day 12, $P \leq 0.040$), hemorrhage (day 12, $P = 0.050$), and after 12 days of CORT administration, due to elevated inflammation ($P < 0.003$). No histopathologic changes ($P \geq 0.120$) were observed in LD-C or ECC treatment birds relative to the CON treatment.

2.4.6 Stress modulates mRNA gene expression of systemic responses

The expression of APPs in the liver and immune cytokines in the bursa of Fabricius were measured to identify systemic markers of a stress response. The administration of CORT modulated mRNA expression of APPs. The expression of *SAA1* mRNA decreased in HD-C treatment birds at the 1 day ($P = 0.010$) and 5 days ($P = 0.033$) time periods relative to CON treatment birds (Figure 2.6D). Ceruloplasmin mRNA expression was elevated ($P \leq 0.032$) at all time points in HD-C treatment birds (Figure 2.6E). Although a trend for increased expression of *TF* was observed after 12 days of HD-C treatment, this change was not defined as significant ($P \geq 0.100$; Figure 2.6F).

Corticosterone administration modulated immune cytokines in the bursa of Fabricius. A decrease ($P = 0.050$) in expression of Interleukin 6 (*IL6*) was observed after 1 day of LD-C administration, but remained unchanged ($P \geq 0.600$) thereafter (Figure 2.6G). Birds treated with HD-C showed increased expression of *IL6* after 5 ($P \leq 0.032$) and 12 ($P \leq 0.001$) days of treatment in comparison to all other treatments (Figure 2.6G). The expression of Transforming Growth Factor β2 (*TGFβ2*) was increased only in birds receiving HD-C after 5 ($P \leq 0.009$) and 12 ($P \leq 0.018$) days of treatment (Figure 2.6H). The expression of Interleukin 1β (*IL1β*) showed no change ($P = 0.590$) due to CORT treatment (Figure 2.6I).

2.5 Discussion

Glucocorticoids, such as CORT, can have varying impacts on host physiologic, metabolic, and immunologic responses [3]. I examined the impact of administering CORT to chickens on hepatic lipid metabolism, metabolite compositions of liver, kidney and breast muscle, as well as a variety of systemic immune and stress responses. Data showed that CORT administration promoted lipid deposition in liver and resulted in distinct metabolite compositions in the liver, kidney, and breast muscle. Moreover, the expression of APPs in the liver and cytokines in bursa of Fabricius were modulated by CORT administration.

Gross and histopathological examinations showed that CORT administration resulted in discolouration of the liver, hepatocellular ballooning, and onset steatosis in birds by 5 days after commencement of high dose CORT administration. Hepatic steatosis develops when the level of fatty acid synthesis exceeds the rate of secretion from the liver [144]. Furthermore, extrahepatic fattening is dependent on intravascular catabolism and release of lipids, which can then be incorporated into adipose tissue [142]. These results are consistent with those of Jiang *et al*. [22], who reported that CORT supplementation resulted in increased liver, cervical, and thigh fat deposition [22].

Gene expression profiles of several hepatic lipogenesis genes were measured and the modulations observed corresponded to the histopathological findings of increased lipid deposition in the liver. These results demonstrated that CORT administration resulted in elevated *FAS* expression after 1, 5, and 12 days of administration in both LD-C and HD-C treatment birds. Acetyl-CoA carboxylase was upregulated in LD-C or HD-C treatment birds after 1 day of CORT administration. Only birds receiving CORT at the low dose continued to show upregulation of *ACC* after 5 and 12 days of treatment. In contrast, *ACC* was down-regulated in HD-C treatment birds after 5 and 12 days of CORT administration. Acetyl-CoA carboxylase is negatively regulated by high cytosol levels of fatty acyl-CoA [29], which may have contributed to the decreased expression of *ACC* that was observed in birds administered CORT at the high dose. The mRNA expression data corresponded to the histopathological data, which showed increased macrovesicular steatosis in HD-C treatment birds. The expression of *ME* was upregulated in birds receiving LD-C and HD-C, but only after 12 days of CORT administration. Unaltered *ME* expression after 1 and 5 days of CORT administration may indicate that the NADPH source needed for lipid synthesis was derived from other metabolic pathways (i.e. pentose phosphate pathway). The levels of *ME* can be altered by fasting and re-feeding states; given that the birds in this study were not starved at the time of sample collection, it is possible that *ME* expression may have been altered by the fed-state of the animals [166]. Birds administered HD-C showed elevated *SREBF1* expression after 1 day of CORT treatment, but like *ACC* expression, showed downregulation after 12 days of CORT administration. Conversely, birds administered LD-C showed elevated *SREBF1* expression only after 12 days of administration. Less is known about the *SREBF* family of proteins in poultry in comparison to mammals where several types of *SREBF* have been identified and have varying functions in the regulation of lipid and cholesterol synthesis [167]. These results are in agreement with previous studies that showed that *FAS*, *ACC*, and *ME* were upregulated under dexamethasone mediated stress, and increased fat deposition under heat stress in chickens [37, 41]. Furthermore, Cai *et al.* [41] showed that dexamethasone administration resulted in higher levels of insulin which was associated with *de novo*

lipogenesis [41, 144]. Collectively, these findings indicate that lipid synthesis was upregulated in birds administered CORT, and that the expression of lipogenesis genes corresponded to gross morphological and histopathological changes.

The liver incorporates lipids into very-low density lipoproteins (VLDL), which are then exported into the bloodstream for delivery to adipose tissue and extrahepatic tissues. Two protein components of VLDL (i.e. *APOB* and *APOC3*) were measured [168]. Additionally, *MTTP* was examined, which is responsible for assembly of VLDL and secretion from the liver [32]. No changes in gene expression of *APOB* were observed with CORT administration. Apolipoprotein B has been indicated as the major protein component of VLDL in chickens [144, 168], although the regulation and secretion of VLDL has not been the subject of much investigation in growing chickens. *In vitro* studies have shown that high concentrations of insulin can enhance lipogenesis while concurrently inhibiting *APOB* synthesis. This can provide some explanation as to why *APOB* gene expression levels were unaltered by CORT administration in the present study [144]. The expression of *APOC3* was increased in birds administered CORT after 1 and 12 days. Little is known about the function of *APOC3* in avian species, and functional significance can only be currently derived from mammalian research. Interestingly, it has been demonstrated that *APOC3* can impair liver clearance of *APOB*, which corresponds to this study's findings on *APOB* expression [169]. Furthermore, *APOC3* has been shown to inhibit hepatic uptake of triglycerides [169]. In the case of this study, elevated *APOC3* can indirectly suggest that hepatocytes were saturated with lipids and further import would not be required. Lastly, *APOC3* has also been shown to promote VLDL assembly and secretion from hepatocytes under lipid rich conditions and this can promote expression of mRNA and function of *MTTP* [170]. This relates to the findings of *MTTP* gene expression, which demonstrated a similar expression pattern as *APOC3*. Both CORT treatments resulted in increased serum cholesterol at all sample time points. This indicates that circulating lipids were readily available for hydrolysis by lipoprotein lipase, a key enzyme necessary for the release of lipids for deposition into adipose tissue and extrahepatic tissues [171]. Only small amounts of lipoprotein lipase are found functionally active in poultry, and previous research has shown its gene expression is not upregulated under stress response [41, 144]. Therefore, lipoprotein lipase was not measured in this study. The administration of CORT supported the up-regulation of genes involved in lipid synthesis in the liver, regardless of the duration of CORT administration (i.e. 1, 5, or 12 days). Increased lipid deposition suggests that energy distribution is affected during periods of stress in chickens, which may have been a contributing factor to reduced weight gain that was observed.

The use of metabolomics is becoming more widespread to investigate disease and identify biomarkers of physiological stress [172, 173]. This study demonstrated distinct metabolite compositions in tissue extracts of liver, kidney, and breast muscle that were associated with CORT administration (i.e. both LD-C and HD-C treatments). These findings collectively demonstrate that physiological stress can alter several metabolic pathways and provides potential directions in the development of novel biomarkers of stress. In this regard, an increase in creatinine was observed in the serum, kidney, and breast muscle of CORT-treated birds, which can indicate muscle breakdown and kidney dysfunction [174, 175]. Heat stress in chickens has been demonstrated to increase creatine kinase activity; this enzyme is responsible for the breakdown of creatine and results in increased creatinine levels [141]. A decrease in anserine and carnosine in muscle tissue of CORT-treated birds was observed, which has been implicated as a marker of muscle dystrophy and altered redox homeostasis in chickens [173, 176]. Furthermore, heat stress studies conducted in chickens and pigs concluded that decreased carnosine in muscle tissue lowered antioxidant capacity and this correlated with poor meat quality [177, 178]. Likewise, high tissue concentrations of taurine are associated with elevated levels of oxidants and can aid in preventing tissue injury and inflammation [179]. A study in mice that examined the metabolome of muscle observed a correlation between increased choline, altered phospholipid metabolism, and membrane breakdown in muscle under high fat conditions [180]. High muscular choline levels observed in the current study may have derived from the hydrolysis of phosphatidylcholine present in VLDL. Given the upregulation of *APOC3* and *MTTP* in CORT-treated animals observed, this may suggest an increased transport of VLDL to extremities of the body, such as muscle tissue. Furthermore, the metabolites betaine and 3-hydroxybutyrate have been identified as discriminating metabolites of fat deposition where betaine was increased while 3-hydroxybutyrate was decreased [145]. Similarly, lipid deposition mediated by CORT treatment in this study resulted in elevated betaine and decreased 3-hydroxybutyrate. Decreased β-alanine and aspartate levels were observed, which may also be associated with CORT-induced lipid synthesis in the liver. Aspartate can be converted into β-alanine, which is subsequently metabolized into acetyl-CoA; however, this would be unnecessary in muscle tissue if lipid deposition is occurring and other energy sources are present [181].

The sugars, fucose and glucose, were observed to be elevated in the breast muscle of CORT-treated birds. Fucose has previously been evaluated as a marker of liver disease where elevated urinary excretion of fucose was suggested to be a marker of impaired glycosylation in the liver [182]. Glucose may have been derived from two sources: (1) the breakdown of glycogen, which has been demonstrated under transport stress previously in chickens [26]; or (2) transport from the liver through the glucose-

alanine cycle. The latter explanation is supported by the observation of increased alanine in the liver; alanine in the liver can be converted to glucose and transported back to muscle. However, glucose transporter 2 was not consistently upregulated in the liver with CORT treatments in this study, which would be expected if glucose transport to muscle was occurring (Appendix A, Figure S1.1). Conclusively, these results demonstrated that CORT-induced stress altered the metabolite profiles of liver, kidney, and breast muscle tissues and supported a stress-induced increase in hepatic lipid synthesis. Importantly, the conspicuous changes in metabolite compositions of tissues observed in birds administered CORT indicates dysregulation of metabolic pathways, and this likely contributed to the impaired weight gain observed.

The expression of both acute phase proteins and immune cytokines were examined as a measure of systemic response. Modulation of APP levels are often associated with inflammatory responses characterized by systemic and metabolic changes [63]. Bacterial agents, trauma, and other inflammatory mediators are often activators of APR. Acute phase proteins can have varying functions, but in general, act to restore homeostasis [63]. The expression of *SAA1* showed consistent decreases in HD-C treatment birds. Serum amyloid A is an apolipoprotein associated with high density lipoprotein and it is generally elevated under APR [183]. A decrease in *SAA1* expression in CORT-treated birds was observed, which may reflect alterations in hepatic lipid metabolism. The expression of *CP* was elevated in HD-C treatment birds at all sample times. Ceruloplasmin levels have been shown to be elevated in poultry proceeding infection with *Eimeria tenella* and *Escherichia coli* [184]. The function of *CP* is to catalyze iron into a ferrous state that can then be incorporated into iron binding proteins such as *TF* [151]. Higher levels of *TF* have been associated with inflammation where increased *IL6* levels and elevated heterophil numbers precede the increase of *TF* [185]. Increases in *TF* have been suggested to function by sequestering iron from bacterial utilization and thereby limit infection. These results however, did not demonstrate substantial increases in *TF* with CORT treatment.

The majority of studies conducted to date have primarily focused on how stress impairs immune response, specifically the generation of antibodies, or adaptive responses in chickens. Shini *et al* [72, 74] examined cytokine and chemokine responses in immune cells, and showed that inflammatory responses can be activated during physiological stress. These results demonstrated upregulation of *IL6* and *TGFβ2* in the bursa of Fabricius in HD-C treatment birds after 5 and 12 days of administration. Interleukin 6 can be produced in response to inflammation, and it is an inducer of APR, which corresponds to this study's findings of modulated APP expression [150]. Furthermore, Zulkifli *et al* [152] demonstrated that *IL6* serum levels were increased by CORT after 3 days of administration and persisted for 7 days. Another

study demonstrated that *IL6*, along with pro-inflammatory cytokines *IL1β* and *IL18*, were upregulated 3 hr post-CORT administration in drinking water (20 mg/L) in peripheral lymphocytes and heterophils, while spleenocytes showed highest levels of these cytokines at 24 hr post-CORT exposure [72, 74]. Although an increase in *IL6* or *IL1β* expression after 1 day of CORT treatment was not observed, mRNA expression in bursa of Fabricius tissue was measured and expression in specific immune cell types was not examined. The bursa of Fabricius is a lymphoid organ unique to chickens and it is responsible for the development of B cells [63]. It is mainly comprised of B cells, some T cells, macrophages, and dendritic cells, which are organized into follicular structures [63]. Increased *IL6* expression within the bursa of Fabricius was observed, which may have been due to tissue degradation [152]. Histopathological examination of the bursa of Fabricius showed that administration of CORT at the high dose induced epithelial atrophy and lymphoid depletion after 5 days of treatment with marked inflammation present after 12 days of CORT administration. Furthermore, the size of the bursa of Fabricius decreased with CORT treatment, and morphology was altered (Appendix A, Figure S1.2). Elevated *TGFβ2* mRNA that was observed in birds treated with CORT may have been due to tissue injury as *TGFβ* expression can be induced during tissue repair [186]. The histopathological changes that were observed after 12 days of CORT administration support induction of inflammatory responses, as well as regulatory responses like *TGFβ2* due to physiological stress. Glucocorticoids can act to both activate and suppress immune responses [71]. Lymphoid depletion and atrophy of the bursa of Fabricius was observed, which is consistent with the suppressive nature of glucocorticoids on immune function with respect to impaired adaptive immune responses. Concurrently, an increased *IL6* and *TGFB* expression was observed, which may have occurred within specific immune cells types as a response to tissue injury occurring. Mammalian research has demonstrated that the suppressive or stimulatory nature of glucocorticoids on inflammatory responses can be dependant on both dose and duration [71].

In summary, CORT-mediated physiological stress resulted in higher expression of lipid synthesis and secretion genes, which led to increased lipid deposition in the liver. Metabolic perturbations observed in the present study demonstrate the power of using metabolomic analysis to identify biomarkers of stress. Further research should collate metabolite changes in tissues to non-destructive markers (i.e. feces, feathers, blood) with the goal of achieving new diagnostic tools to better monitor on-farm stress. These findings also showed that CORT treatment altered hepatic lipid metabolism, metabolite compositions in tissues, and APR after 1, 5, or 12 days of treatment. Likewise, modulations to cytokine expression in the bursa of Fabricius occurred after 5 and 12 days of CORT treatment likely due to atrophy and other tissue damage. Collectively, the altered metabolic responses, APR induction, and

immune modulations induced by CORT treatment likely contributed to the impaired weigh gain observed in birds. This has significant ramifications for poultry production, and additional research is required to investigate how poultry production stressors (i.e. thermal, social, etc.) can modulate immune function by examining cytokine profiles, immune cell trafficking, and paradigm immune responses. Crucially, obtaining a better understanding of how production stressors impact host immune and metabolic function is necessary to the identify and implement effective management regimes to enhance chicken health.

2.6 Figures

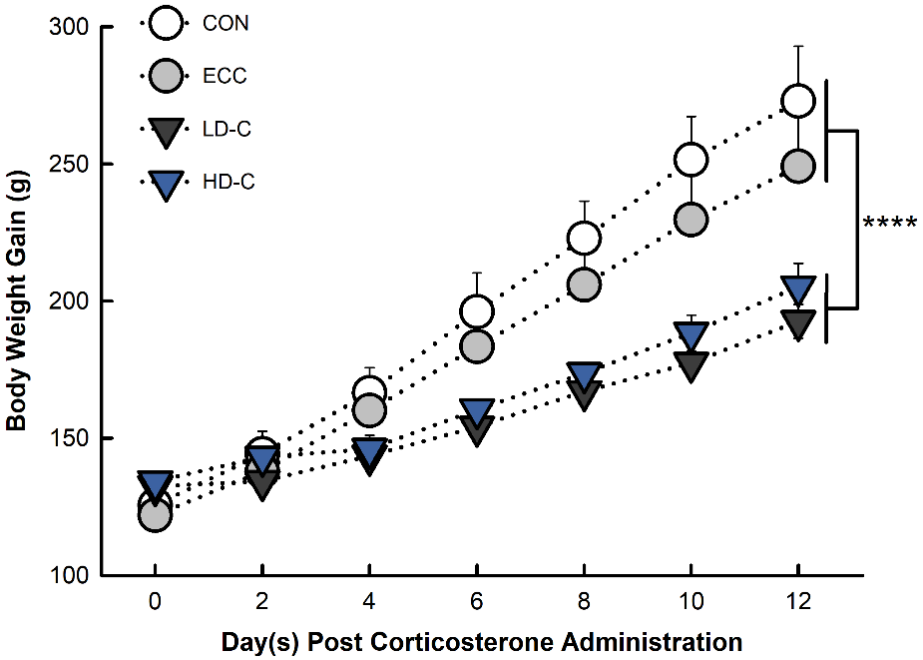

Figure 2.1 Effect of CORT treatment on body weight gain. Birds were administered standard drinking water (CON treatment), 0.2% ethanol in drinking water (ECC treatment), 10 mg/L CORT (LD-C treatment), or 30 mg/L CORT (HD-C treatment). Vertical lines associated with markers represent standard error of the means (n = 3); markers without vertical lines indicates marker is obscuring the standard error mean. **** $P < 0.001$.

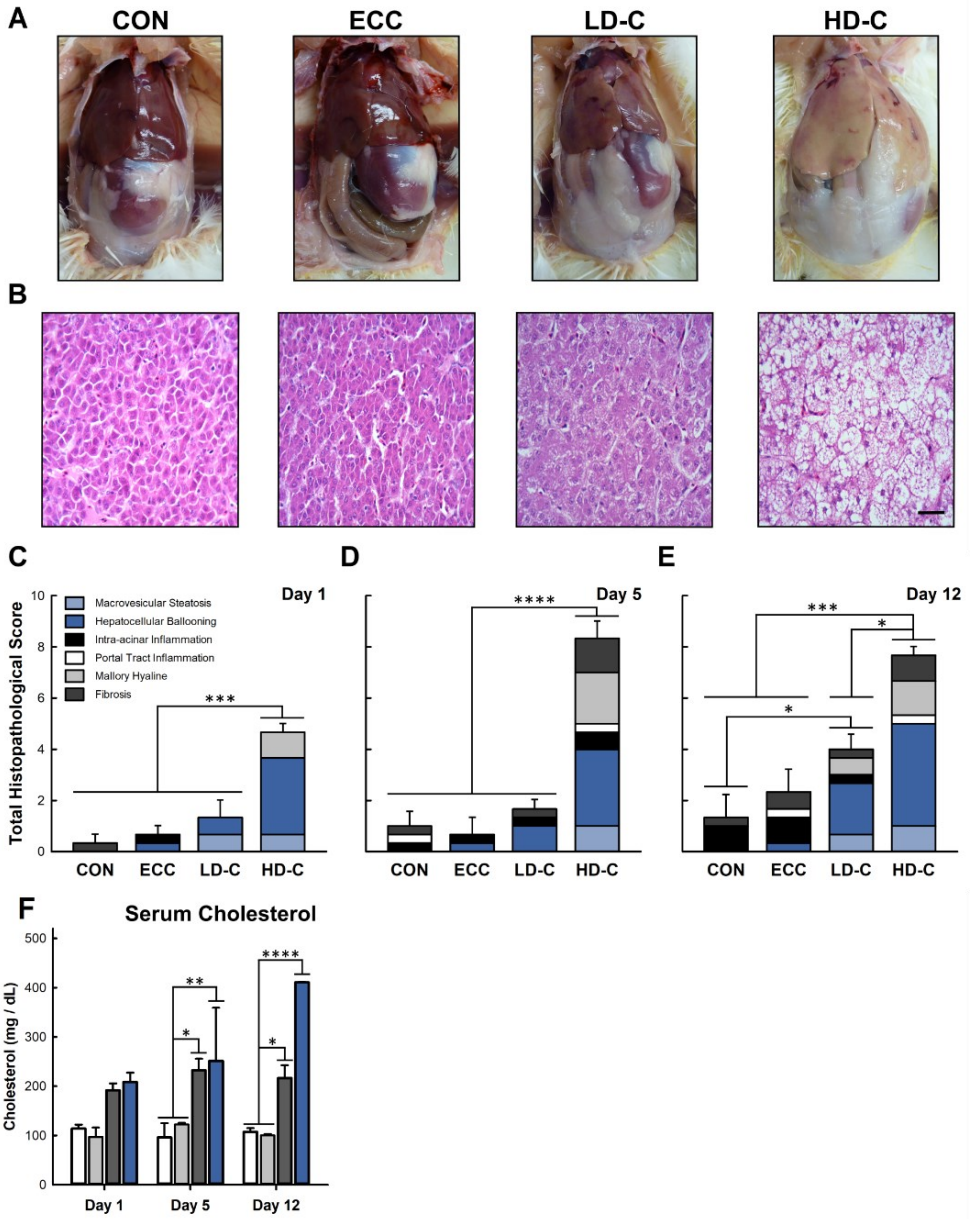

Figure 2.2 Effect of CORT treatment on host physiology and histopathology of the liver. Birds were administered standard drinking water (CON treatment), 0.2% ethanol drinking water (ECC treatment), 10 mg/L CORT (LD-C treatment), or 30 mg/L CORT (HD-C treatment); birds were euthanized and sampled at 1, 5, or 12 days post continual administration of CORT. (A) Gross morphology of liver and adipose tissue 12 days post-CORT administration. (B) Histopathological changes in liver tissue at 12 days post-CORT administration. Bar is 20 µm. (C-E) Total histopathological scores of liver. (C) 1 day post-CORT administration. (D) 5 days post-CORT administration. (E) 12 days post-CORT administration. (F) Serum cholesterol. Vertical lines associated with histogram bars represent standard error of the means (n = 3). * $P < 0.050$, ** $P < 0.010$, *** $P < 0.001$, and **** $P < 0.0001$.

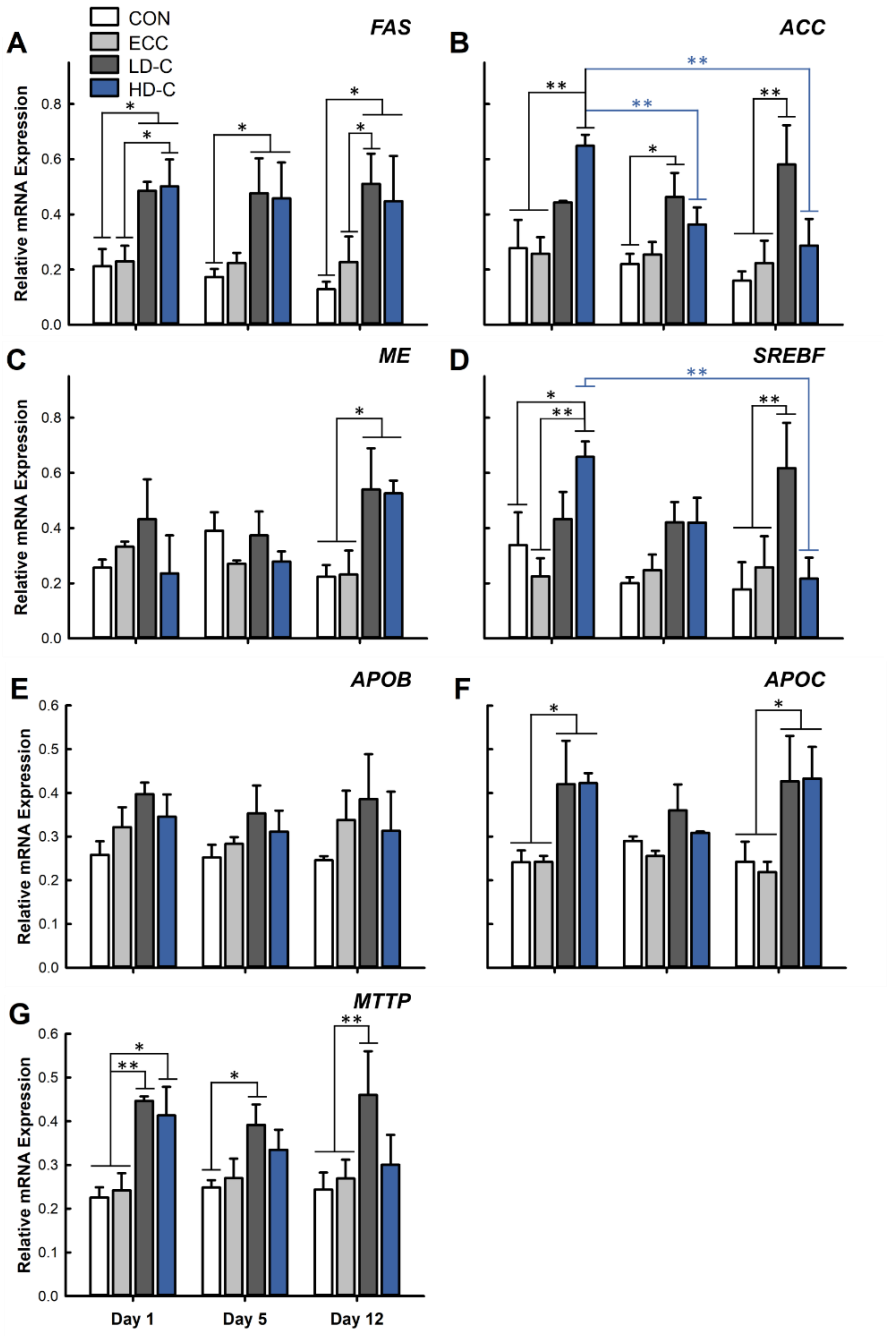

Figure 2.3 Corticosterone treatment modulates mRNA gene expression of lipid metabolism genes within the liver. Birds were administered standard drinking water (CON treatment), 0.2% ethanol drinking water (ECC treatment), 10 mg/L CORT (LD-C treatment), or 30 mg/L CORT (HD-C treatment); birds were euthanized and sampled at 1, 5, or 12 days post-CORT administration. (A-G) Relative expression of mRNA transcripts. (A) *FAS.* (B) *ACC*. (C) *ME*. (D) *SREBF1.* (E) *APOB*. (F) *APOC3.* (G) *MTTP*. Vertical lines associated with histogram bars represent standard error of the means (n = 3). * $P < 0.050$, ** $P < 0.010$, *** $P < 0.001$, and **** $P < 0.0001$.

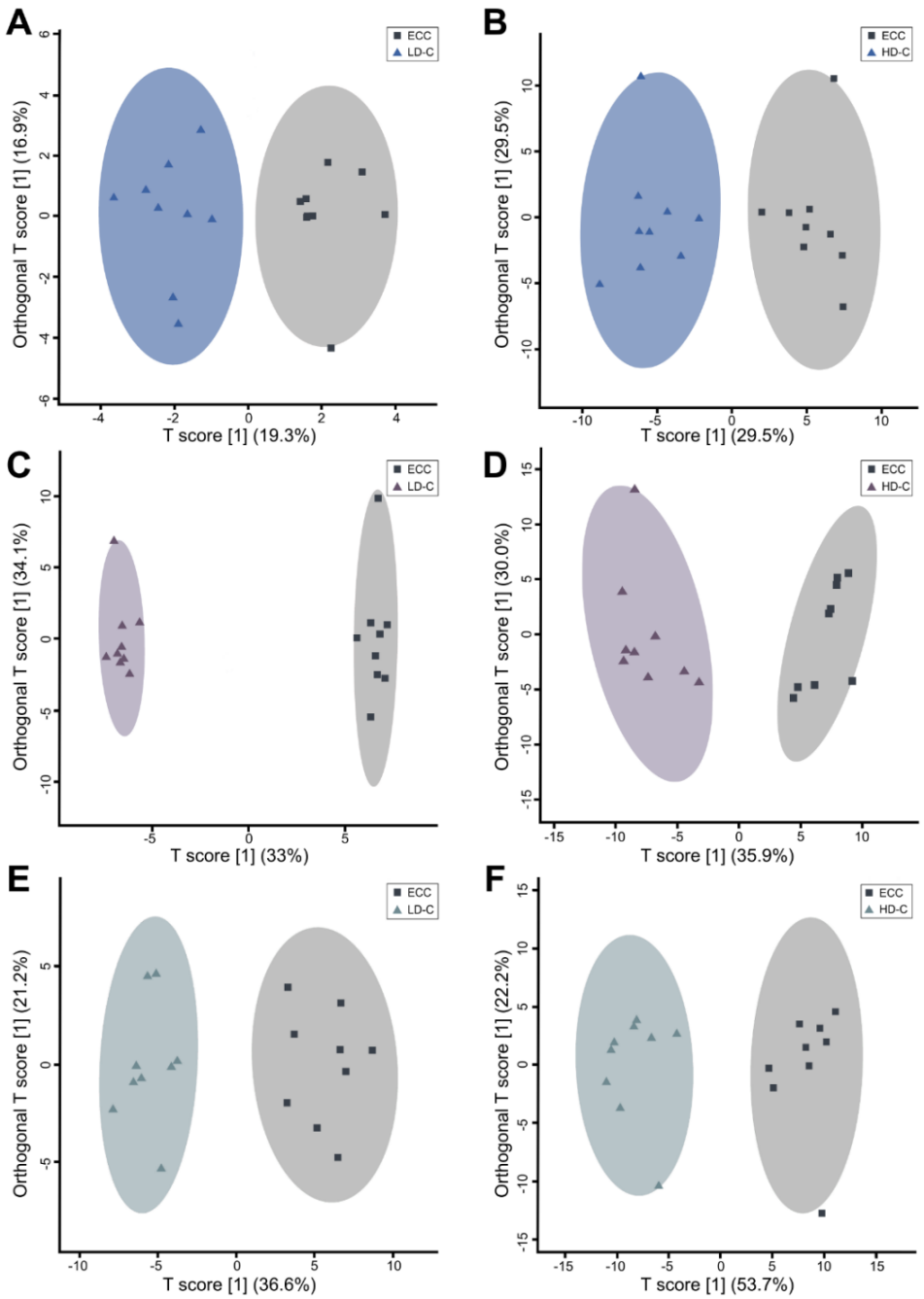

Figure 2.4 Metabolite profiles of kidney, liver, and breast muscle. Orthogonal partial least squares discriminant analysis (OPLS-DA) plots showing supervised separation for (A-B) kidney tissue, (C-D) liver tissue, and (E-F) breast muscle tissue where (A,C,E) shows separation between birds administered 0.2% ethanol in drinking water (ECC treatment) or 10 mg/L CORT drinking water (LD-C treatment), and (B,D,F) shows separation between ECC treatment birds or 30 mg/L CORT of drinking water (HD-C treatment). Each square or triangle represents one bird; data was plotted using metabolites identified to be significant by MW and/or VIAVC. n = 9 birds per treatment.

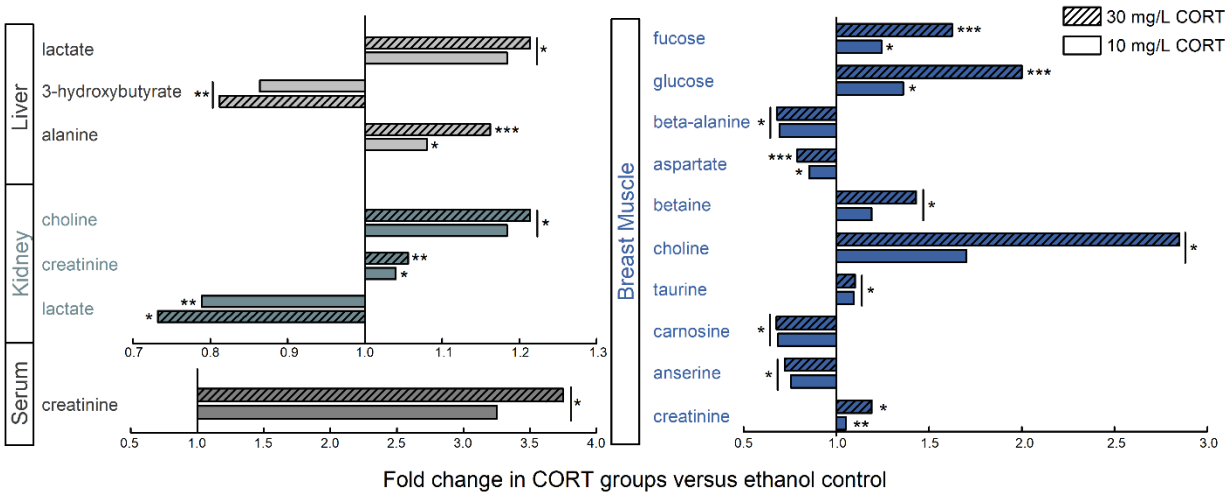

Figure 2.5 Fold-change of discriminating metabolites of CORT treatment. Metabolites identified by ^{1}H-NMR (liver, kidney, and breast muscle) and VetTest chemistry analyzer (serum). Individual metabolites and sample type are indicated; bars indicate fold change of normalized metabolome of birds receiving either 10 mg (LD-C treatment) or 30 mg (HD-C treatment) divided by the ethanol (ECC) treatment birds. n = 9 birds per treatment. * $P < 0.050$, ** $P < 0.010$, *** $P < 0.001$.

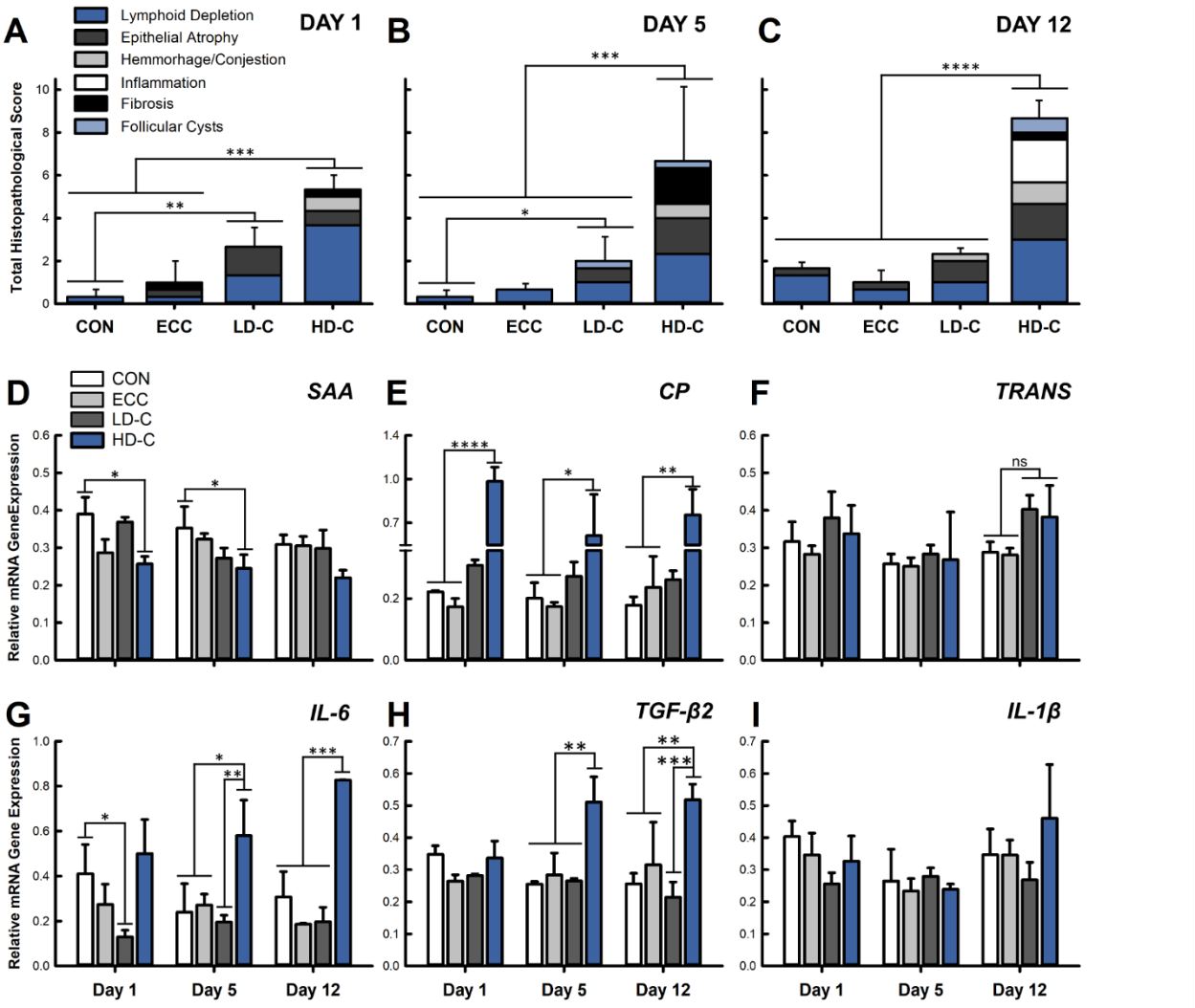

Figure 2.6 Effect of CORT treatment on systemic responses. Birds were administered standard drinking water (CON treatment), 0.2% ethanol drinking water (ECC treatment), 10 mg/L of CORT (LD-C treatment), or 30 mg/L of CORT (HD-C treatment); birds were euthanized and sampled at 1, 5, or 12 days post-CORT administration. (A-C) Total histopathologic change scores for bursa of Fabricius for (A) 1 day, (B) 5 days, or (C) 12 days post-CORT administration. (D-I) Relative mRNA gene expression in (D-F) liver and (G-I) bursa of Fabricius. (D) *SAA1.* (E) *CP.* (F) *TF.* (G) *IL6.* (H) *TGFβ2.* (I) *IL1β.* Vertical lines associated with histogram bars represent standard error of the means (n = 3). * $P < 0.050$, ** $P < 0.010$, *** $P < 0.001$, and **** $P < 0.0001$.

Chapter 3: Physiological stress mediated by corticosterone administration alter intestinal bacterial communities and increased the relative abundance of *Clostridium perfringens* in the small intestine of chickens[2]

3.1 Abstract

A model of physiological stress mediated by the administration of corticosterone (CORT) was used to investigate the impact of stress on the intestinal microbiota of chickens. Birds were administered CORT in their drinking water at 0, 10 (low dose CORT; LDC), and 30 (high dose CORT; HDC) mg/L. Digesta from the small intestine and ceca were examined after 1, 5, and 12 days post-initiation of CORT administration by 16S rRNA gene sequencing. A decrease in phylogenetic diversity and altered composition of bacteria were observed for HDC in the small intestine. Analysis by ANOVA-Like Differential Expression 2 (ALDEx2) showed that densities of *Clostridium sensu stricto* 1 bacteria were increased in the small intestine for LDC and HDC. Quantitative PCR confirmed that CORT administration increased densities of *Clostridium perfringens* in the small intestine, but only HDC was associated with increased densities of the bacterium in ceca. Predictive functional analysis by Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2 (PICRUSt2) showed pathways of carbohydrate metabolism to be enriched with CORT, and amino acid synthesis to be enriched in control birds in the small intestine. In conclusion, physiological stress mediated by CORT modulated bacterial communities in the small intestine and increased densities of *C. perfringens*. This implicates stress as an important mediator of this important enteric pathogen in poultry.

3.2 Introduction

The activation of the hypothalamus–pituitary–adrenal axis in poultry results in the production of corticosterone (CORT), the primary glucocorticoid stress hormone. The production of CORT relays numerous impacts to bird health, including modifications to bird metabolism and immune function [3]. Impairment to weight gain and muscle assimilation are some of the reported effects of physiological stress on bird performance [25, 38]. My previous research showed CORT administration to birds promoted hepatic lipid synthesis and distinctly alter metabolite profiles of liver, kidney, and breast muscle tissue [36]. It is well recognized that glucocorticoids impart many immunomodulatory effects on mammalian and avian species [4]. In poultry, it has been shown that short term CORT exposure can incite inflammatory responses, whereas chronic CORT exposure stimulates immunoregulatory responses

[2] A version of this chapter has been previously published: Zaytsoff, S.J.M.; Uwiera, R.R.E.; Inglis, G.D. Physiological stress mediated by corticosterone administration alters intestinal bacterial communities and increases the relative abundance of *Clostridium perfringens* in the small intestine of chickens. *Microorganisms* **2020**, *8*, 1518.

[4]. The study of the intestinal microbiota of poultry and its relationship to host health and production performance is of great interest. Considering the modifications that physiological stress imparts on the host, and the bi-directional influence of the host–microorganism relationship, investigation of the poultry microbiota under physiological stress is warranted.

The intestinal microbiota has been shown to influence nutrition uptake, immune development, and provide colonization resistance against incoming intestinal pathogens [187, 188]. Various factors can alter the composition of the microbiota in chickens, including the presence of antibiotics in feed, the age and sex of the animal, housing practices, and diet [100]. Modern poultry production can subject birds to varying stressful experiences and can result in negative health outcomes [3]. The influence of physiological stress on the poultry microbiota is beginning to be explored. For example, heat stress has been shown to affect the bacterial community composition of ileal and cecal digesta and feces in chickens [108-110]. Bacterial communities of the intestinal tract are sensitive to stress, and various alterations to the enteric environment can result in changes in the microbiota [107]. For example, social stress in mice altered the stability of the intestinal microbiota, reduced diversity, and promoted translocation of bacteria [111]. Physiological stress can alter the morphology of the intestine, promote digesta transit and mucus secretion, and alter mucosal permeability [107]. All of these parameters can influence the enteric environment and consequently disrupt the normal microbiota [107, 189]. However, the mechanisms by which stress influences the microbiota and enteric disease remain speculative, particularly in chickens.

Alterations to the microbiota can affect host health and increase predisposition to enteric disease. Stress-induced modifications to the intestinal microbiota have been associated with increased cytokine production and the modulation of immune activity [111]. Putative beneficial bacteria (e.g., *Lactobacillus* spp.) have been shown to decrease in abundance following exposure to physiological stress [107, 112]. Stress can also lead to increased colonization by foodborne pathogens such as *Escherichia coli* and *Salmonella* spp. [107, 190]. Necrotic enteritis (NE) is a disease of the small intestine in chickens incited by *Clostridium perfringens*. Despite the manifestation of NE in the small intestine, afflicted birds can exhibit a perturbation of bacterial communities in ceca, although the mechanisms are unknown [191]. Notably, correlating enteric bacterial colonization patterns with disease may lead to the discovery of microbial biomarkers that may aid in the development of diagnostic methods and provide information on key factors that contribute to disease initiation and development.

Advances in DNA sequencing technology have promulgated an interest in defining how the microbiota may be modified within the intestine and influence host health. Given the ubiquitous nature

of stress in poultry production, the current study examined how the enteric microbiota was altered in a chicken model of physiological stress mediated by the administration of the glucocorticoid stress hormone, CORT. I utilized this model of exogenous CORT administration as it consistently elevates levels of CORT and mediates a stress response in a prescribed manner [154]. Furthermore, this model has been used on numerous occasions to examine how stress alters host functions in poultry [44, 45, 154, 192]. I hypothesized that the structure of bacterial communities in the small intestine and ceca of chickens administered CORT will be altered relative to the diversity and composition of the bacteria in birds not treated with CORT, and that resident populations of *C. perfringens* will be favored in birds with reduced bacterial diversity due to CORT administration. The objectives were to (1) incite physiological stress in chickens via administration of CORT; (2) measure the richness, composition, diversity, and structure of bacterial communities in the small intestine and ceca of birds ± CORT; and (3) ascertain the effects of physiologic stress on densities of the pathogen, *C. perfringens*.

3.3 Materials and Methods

3.3.1 Ethics statement

The study was conducted in strict accordance with the recommendations specified in the Canadian Council on Animal Care Guidelines. The project study was reviewed and approved by the Lethbridge Research and Development Centre Animal Care Committee (Animal Use Protocol #1526) before the commencement of the research.

3.3.2 Study design

The experiment was arranged as a completely randomized design with four levels of stress treatment and three levels of time (i.e., 4 × 3 factorial design with three biological replicates). The four stress treatments were: control (CON; untreated drinking water); ethanol carrier control (ECC; 0.2% ethanol in drinking water); low dose CORT (LDC; 10 mg CORT/L of drinking water); and high dose CORT (HDC; 30 mg CORT/L of drinking water). Birds were terminally sampled from each stress treatment at 1, 5, and 12 days after the initiation of CORT treatment (12 treatment groups, n = 3 per treatment). The three biological replicates were completed on separate occasions to ensure independence, and the experiment was comprised of 36 birds in total.

3.3.3 Animal husbandry

Specific-pathogen-free white leghorn chickens' eggs were obtained from the Canadian Food Inspection Agency (Ottawa, ON, CA). Eggs were incubated and hatched as previously described [36]. Chicks (1-day-old) were acclimatized in a group within one large animal pen for 10 days with free access to a brooder (Brinsea Products Inc., Titusville, FL, USA). At 11-days-of-age, birds were randomly assigned

to the four stress treatments and housed in groups of four within an individually ventilated cage system (Techniplast, Montreal, QC, CA) as previously described [36]. Each animal cage contained an additional companion bird to ensure that no birds were left socially isolated. Birds were provided free access to a non-medicated starter diet (Hi-Pro Feeds, Lethbridge, AB, CA [193]) and water at all times. Birds were maintained on a 12 hr light: 12 hr dark cycle, and were weighed daily. Corticosterone administration commenced in birds that were 14-days-of-age as described previously [36]. Corticosterone (10 or 30 mg) was dissolved in 2.0 mL of absolute ethanol and added to 1 L of drinking water. Water containing CORT was prepared each morning and changed twice during each day.

3.3.4 Sample collection

One bird from each treatment was randomly selected at each sample time. Birds were anaesthetized with isoflurane (5% isoflurane; 1 L O_2/min) and humanely euthanized by cervical dislocation. The small intestine and ceca were aseptically removed and longitudinally opened using a sterile blade. Digesta in the intestinal lumen was removed from the small intestine at the jejunum–ileum junction (Meckel's diverticulum) and from ceca using a sterile wooden splint, and stored at -80°C until processing.

3.3.5 DNA extraction and 16S rRNA gene sequencing

Bacterial genomic DNA from intestinal digesta of the small intestine (jejunum–ileum) and ceca was extracted using a QIAamp Fast DNA Stool Mini Kit (Qiagen, Inc., Toronto, ON, CA). DNA from digesta was sent to Genome Quebec (Montreal, Quebec, CA) for library preparation and 16S rRNA gene sequencing. The V3-V4 region of the 16S rRNA gene was amplified using PCR primers 341F: 5'-CCTACGGGNGGWGCAG and 805R: 5'-GACTACHVGGGTATCTAATCC. The PCR product was then sequenced using a MiSeq (Illumina®, San Diego, CA, USA), obtaining 250 bp paired-end reads.

3.3.6 Sequencing data analysis

Quantitative Insights Into Microbial Ecology 2 (QIIME 2™, version 2019.10; [194]) was used to facilitate sequencing analysis while filtering of low quality reads (quality score <20), trimming sequences, and paired-end reads were joined using DADA2. Joined reads were grouped into exact amplicon sequence variants (ASVs, *n* = 1265) where taxonomy was classified using the SILVA bacteria reference database (release version 132) [195]. Low count reads, mitochondrial sequences, and chloroplast sequences were filtered out, and analysis was conducted on 1150 bacterial ASVs. Sampling depth for the small intestine and ceca was set to 15,000 and 23,000 reads, respectively. Three samples from the small intestine were omitted from analysis due to low sequence counts (1 from CON-day 5 and 2 from LDC-day 1 and 12). There was no difference among the sample times, and analysis of stress

treatments was thus averaged over sample days. Core metric analysis was implemented in QIIME2 to obtain alpha (Faith's phylogenetic and Shannon's diversity) and beta (Jaccard, Bray–Curtis, and weighted UniFrac distance) diversity results. Alpha diversity was analyzed by pairwise comparisons of the Kruskal–Wallis test. Beta diversity was analyzed by pairwise permutational multivariate analysis of variance (PERMANOVA) [196]. A Benjamini and Hochberg correction was applied to both pairwise alpha and beta diversity tests when corrected P-values were < 0.050 relative to both the CON and ECC treatments. A table of ASVs was exported from QIIME2 and used to generate compositional bar plots and heatmaps in GraphPad Prism (version 8.4.2). The R package ALDEx2 plugin was utilized in QIIME2 to differentiate significant taxa ($q < 0.10$) between control birds (CON and ECC groups) and CORT treatment birds (LDC and HDC) [197]. Percent abundance of significant taxa (*Clostridium sensu stricto* 1) was normalized, and one-way ANOVA was applied using the GraphPad Prism software (La Jolla, CA, USA, version 8.2.4) with a multiple comparison Tukey's significant difference test. Results (LDC and HDC) were deemed significant when $P < 0.050$ relative to CON and ECC. Data in figures were plotted as mean ± standard error of mean (SEM). Predictive functional analysis was completed by using the PICRUSt2 plugin for QIIME2 [198]. The generated Kyoto Encyclopedia of Genes and Genomes (KEGG) ortholog and MetaCyc pathway abundance tables were analyzed by ALDEx2 to identify significant features that differed between the control treatment birds (CON and ECC) and those administered CORT (LDC and HDC).

3.3.7 Quantitative PCR

The basic quantitative PCR (qPCR) protocol described by Zaytsoff et al. [193] was used. Bacterial genomic DNA from the small intestinal and cecal digesta was extracted as indicated above. For standard curve generation, genomic DNA from a pure culture of *C. perfringens* was extracted using DNeasy Blood and Tissue Kit (Qiagen, Inc., Toronto, ON, CA). Briefly, cell biomass was lysed using enzymatic lysis buffer (20 mM Tris·Cl, pH 8.0; 2 mM sodium EDTA; 1.2% Triton X-100; 20 mg/mL lysozyme) instead of the tissue lysis solution (Qiagen, Inc., Buffer ATL) supplied in the kit. A standard curve of known copies of 16S rDNA specific to *C. perfringens* was generated with DNA amplified from the extracted DNA using CP1.2 primers F: 5'-AAAGATGGCATCATCATTCAAC and R: 5'-TACCGTCATTATCTTCCCCAAA [199]. Amplicons were visualized in a 2% agarose gel, and the amplicon was extracted using a QIAquick Gel Extraction Kit (Qiagen, Inc., Toronto, ON, CA). To generate a standard curve of known gene copies, the gel-extracted DNA was quantified fluorometrically using Qubit™ 2 (Life Technologies, Burlington, ON, CA), and copies of genes were normalized to 10^7 copies/µL based on concentration, amplicon size, and nucleotide weight. A standard curve was generated by diluting DNA in a 10-fold dilution series and amplifying *C. perfringens* 16S rDNA using CP1.2 primers. Quantitative PCR was used to measure *C.*

perfringens densities in the small intestinal and cecal digesta relative to the standard curve and normalized by the weight of the sample. Each reaction contained 5.0 µL Quantitect SYBR green master mix (Qiagen, Inc., Toronto, ON, CA), 0.5 µL of each primer (10 µM), 1.0 µL bovine serum albumin (1 mg/mL), 2.0 µL DNase-free water, and 1.0 µL template DNA. Reactions conditions were: 95 °C for 15 min; and 40 cycles of 95 °C for 15 s, 55 °C for 30 s, and 72 °C for 30 s; and melt curve analysis from 55–95 °C. An ABI7900HT thermocycler (Applied Biosystems, Carlsbad, CA, USA) was used. Reactions were run in triplicate, and the mean of the three observations was calculated. The qPCR results were assessed for normality using GraphPad Prism software (La Jolla, CA, USA, version 8.4.2), and analyzed by one-way ANOVA to determine differences among CORT treatments. A multiple comparison Tukey's significant difference test was applied; results (LDC and HDC) with $P < 0.050$ were considered significant relative to both CON and ECC. Data are represented as mean ± SEM.

3.3.8 Characterization of *Clostridium perfringens*

Fecal samples were collected from birds at 16-days-of-age, serially diluted, and the suspension spread on Columbia agar supplemented with 10% sheep's blood (Difco, Frankon Lakes, NJ, USA). Putative colonies for *C. perfringens* were re-streaked for biomass and extracted using a DNeasy Blood and Tissue Kit (Qiagen, Inc., Toronto, ON, CA) as described above. Putative *C. perfringens* isolates were subjected to PCR using CP1.2 primers and NetB toxin gene primers (F: 5'-AAATATACTTCTAGTGATACCGCTTCACA-3'; R: 5'-GAGGATCTTCAATAAATGTTCCACTTAA-3') [128]. Reactions conditions were: 95 °C for 10 min; and 35 cycles of 95 °C for 15 s, 55 °C for 30 s, and 72 °C for 30 s. PCR products were visualized on a 2% agarose gel to identify strains that were ± *C. perfringens* and/or NetB positive.

3.3.9 Data availability

The raw sequencing reads were submitted to the Sequencing Read Archive of NCBI under BioProject accession PRJNA647907. Sample metadata can be found in Appendix B, Table S2.1.

3.4 Results

3.4.1 Corticosterone treatment alters the intestinal microbiota composition

Alpha diversity was assessed through Shannon's and Faith's phylogenetic diversity indices in the small intestine and ceca. No changes ($P \geq 0.240$) in Shannon's diversity were observed in the small intestine or ceca (Figure 3.1A,B). Faith's phylogenetic diversity decreased in the small intestine ($P \leq 0.028$) but not in ceca ($P \geq 0.760$) of HDC treatment birds in comparison to the CON and ECC treatments (Figure 3.1C,D). No changes ($P \geq 0.150$) in Faith's phylogenetic diversity were observed in the small intestine and ceca with LDC treatment relative to CON and ECC treatment birds. Community similarity

was evaluated by Jaccard, Bray-Curtis, and weighted UniFrac distances. The HDC treatment in the small intestine diverged from both CON and ECC treatments with Jaccard ($P \leq 0.003$), Bray–Curtis ($P \leq 0.030$), and weighted UniFrac ($P \leq 0.006$) distances (Figure 3.2A–C). Sequences of the *Clostridium sensu stricto* 1 genus were higher in LDC and HDC treatment birds (Figure 3.2D). Heatmap visualization of ASVs in the small intestine showed extensive loss of taxa within the HDC treatment birds (Figure 3.2E). In ceca, bacterial community similarity was changed with HDC treatment relative to CON and ECC treatments, as measured by Jaccard's ($P \leq 0.048$) (Figure 3.3A). Bray–Curtis ($P \geq 0.102$) and weighted UniFrac ($P \geq 0.120$) distance did not demonstrate any compositional changes among treatments (Figure 3.3B,C). Bacteria within the cecal digesta possessed a relatively stable taxonomic distribution, and no single taxon dominated among any of the treatments (Figure 3.3D,E).

3.4.2 Corticosterone treatment increases *Clostridium perfringens* densities

ALDEx2 analysis was applied to both the small intestine and cecal bacterial community data to identify taxa that differed between non-CORT treatment (CON and ECC) and CORT-treatment (LDC and HDC) birds. Analysis of small intestinal communities identified two ASVs that were both classified in the genus *Clostridium sensu stricto* 1 (Figure 3.4A). The abundance of *Clostridium sensu stricto 1* ASVs in LDC ($P \leq 0.015$) and HDC ($P < 0.001$) treatment birds was higher than CON and ECC treatment birds (Figure 3.4B). In contrast to the small intestine, no ASVs were determined to be altered by ALDEx2 analysis in ceca (Figure 3.4C). However, the abundance of *Clostridium sensu stricto 1* ASVs in ceca was higher ($P \leq 0.017$) in HDC treatment birds in comparison to CON and ECC treatment birds (Figure 3.4D). The Basic Local Alignment Search Tool (BLAST) analysis of the two ASVs that were more abundant in the small intestine of LDC and HDC treatment birds by ALDEx2 analysis revealed a strong sequence match (99.76% and 100%) with *C. perfringens*. Furthermore, several isolates recovered from feces were identified as *C. perfringens* by PCR; no isolates were positive for the NetB toxin gene. Thus, qPCR was performed on DNA extracted from small intestinal and cecal digesta. Densities of *C. perfringens* determined by qPCR corresponded to Illumina sequencing results. In this regard, the small intestine contained higher densities ($P < 0.001$) of *C. perfringens* in CORT treatment birds (both LDC and HDC) in comparison to birds not administered CORT (CON and ECC; Figure 3.4E). Moreover, only the HDC treatment birds showed higher densities ($P < 0.001$) of *C. perfringens* in ceca (Figure 3.4F).

3.4.3 Predictive functional analysis is altered with corticosterone administration

Predictive functional outputs (KEGG Orthologs (KOs) and MetaCyc pathway abundance) from PICRUSt2 were analyzed using ALDEx2 to identify significant features among control (CON and ECC) and CORT treatment (LDC and HDC) birds. ALDEx2 analysis identified 430 and 125 KOs that were altered

between control and CORT treatment birds in the small intestine and ceca, respectively (Figure 3.5A,B). Pathway abundance in the small intestine differed for 32 pathways (Figure 3.5C). In general, carbohydrate biosynthesis and degradation were enriched in CORT administered birds, while amino acid biosynthesis was more abundant in control birds. ALDEx2 analysis of pathway abundance in ceca identified 15 pathways that were altered between the control and CORT treatment birds (Figure 3.5D).

3.5 Discussion

The primary objective of the current study was to understand the degree to which bacterial communities are altered in the small intestine and ceca of chickens when subjected to physiological stress mediated by CORT administration. The microbiota in the small intestine was more sensitive to CORT-induced changes, and both the diversity and composition of bacterial communities were affected. The microbiota in ceca was affected by CORT administration to a lesser degree than in the small intestine, although qualitative changes in the composition of the bacterial community were observed. Significantly, bacterial sequences identified as *Clostridium sensu stricto* 1 increased in the small intestine of birds administered CORT, and qPCR analysis indicated that the increase in *Clostridium sensu stricto* 1 sequences was attributed to *C. perfringens*, the incitant of NE in chickens.

The high bacterial richness in the intestine is associated with positive health benefits, and a functionally diverse microbiota is better able to occupy niches inhabited by pathogens [200]. There are many factors that can influence bacterial richness in chickens, especially when accounting for the variability in bacterial development in young birds [6, 100]. A decrease in intra-bird phylogenetic diversity with the HDC treatment in the small intestine. The induction of a social stressor in mice resulted in the alpha diversity of bacterial communities in ceca to be decreased [111]. Likewise, social conflict in wild male birds was associated with reduced bacterial phylogenetic diversity in fecal samples [201]. A reduction in diversity may be due to innate immune activation as a result of acute stimulation of the hypothalamic–pituitary–adrenal axis [202]. In chickens, short-term CORT administration has been shown to increase the expression of some inflammatory cytokine and chemokines [4, 72]. The modulation of an immune response by CORT, taken together with the instability of the microbiota in young birds, may have contributed to a reduction in diversity that was observed in HDC birds. Moreover, the overgrowth of *C. perfringens* may have hindered the restoration of bacterial diversity.

In the present study, bacterial communities in the small intestine of chickens administered HDC clustered separately from other treatments. In ceca, qualitative changes in bacterial communities in birds administered HDC was observed. These results correspond with previous reports that demonstrated that bacterial communities were affected by high temperatures during the rearing of

broiler and layer chickens [108, 110]. The host-microorganism relationship is complex and functions in a bi-directional manner. It is uncertain whether stress altered the host in a manner that alters bacterial communities or the modulations to bacterial communities, consequently, modified the host. Various factors induced by stress may be driving changes in the microbiota. Intestinal morphology can be altered by heat stress, including decreased crypt depth [107]. The integrity of the intestinal structure is imperative to maintain barrier function and prevent the translocation of bacteria [107, 111]. A study investigating the impacts of a social stressor in mice showed that bacteria could translocate to secondary lymphoid tissue under conditions of stress [203]. The intestinal mucus barrier is another factor that can be modulated by physiological stress. Intestinal glycans have been shown to regulate bacterial communities and limit the number of microorganisms that access the epithelium [204]. My previous research has shown that stress induced by CORT increased mucin quantities in the small intestine of chickens [193]. Additionally, my research has demonstrated that *C. perfringens* colonization in the presence and absence of CORT administration could modify the composition of intestinal mucus glycans [193, 205]. This verifies that bacteria, and likely, the shift in bacterial communities, can result in changes in the host. I have also previously reported that CORT administration can alter the expression of tight junction proteins in the small intestine, as well as immune cytokines in the spleen and thymus [193]. Although my previous work did not examine the microbiota, it is likely that changes in the intestinal bacterial community occurred as was observed in the current study, and this may have modulated host functions.

The microbiota in the small intestine was affected to a greater extent than in ceca of birds administered CORT. This may be due to the substantially higher diversity of bacteria within ceca. In general, chickens have decreased bacterial diversity in the intestine in comparison to other species [206]. Although the reason for this is not fully understood, it has been attributed to the relatively fast transit time of digesta in chickens [206]. This study confirmed a substantially higher alpha diversity in ceca in comparison to the small intestine. Bacterial diversity is an important driver of immune development, and delayed colonization by commensal bacteria or decreased bacterial diversity due to stress can permanently alter T-cell receptor repertoires and, consequently, result in aberrant immune reactions to commensal microbiota [207]. These concepts enforce the significance of promoting a diverse microbiota to aid in host health. It is noteworthy that the current study was coordinated in a research facility with high hygiene standards, and birds thus had limited exposure to microorganisms from the environment. The maintenance of birds in the research facility likely contributed to the low abundance of *Lactobacillus* spp. that was observed in the present study, which are considered to be a

key group of bacteria in the small intestine of poultry [100]. Moreover, study findings accentuate the importance of exposing birds to microorganisms after hatching to expedite enteric bacterial diversity, stability, and colonization resistance.

Corticosterone administration significantly affected the abundance of bacteria within the genus, *Clostridium sensu stricto* 1, to which *C. perfringens* belongs. Considering the importance of NE incited by *C. perfringens* on poultry production worldwide, qPCR was completed to examine the impacts of stress on *C. perfringens* densities. In this regard, CORT administration was associated with increased densities of *C. perfringens* in the small intestine and ceca (only with HDC treatment). *Clostridium perfringens* is an endospore-forming taxon, so it is not unexpected that the birds became colonized with the bacterium via the ingestion of substrates during hatching and present within the rearing environment [208, 209]. Birds were not administered any antimicrobials in the current study, which may have been a contributing factor to *C. perfringens* overgrowth. For example, chickens on organic farms not administered antimicrobials exhibit higher colonization rates by *C. perfringens* [100, 210]. These results are in agreement with other studies in poultry that have demonstrated that potential stressors experienced during production, such as heat and cold temperature exposures and high stocking densities, can be associated with increased densities of *C. perfringens* [130-132]. It is unclear from the results of the current study, as well as previous reports, whether increased *C. perfringens* densities may have been a contributor to altered bacterial diversity observed in the current study. Previous reports linking stress with increased *C. perfringens* densities did not examine the intestinal microbiota [130-132]. The altered structure of bacterial communities that was observed, which coincided with increased *C. perfringens,* are not mutually exclusive factors as *C. perfringens* only increased in birds receiving CORT treatment. In the absence of stress, *C. perfringens* infection has previously been shown to affect the cecal microbiota [191]. It is noteworthy that birds in the current study did not show any overt signs of infection consistent with NE. Additionally, the *C. perfringens* identified in the current study was not positive for the NetB toxin gene. As the NetB toxin is considered to be an important virulence factor, it is unlikely that the *C. perfringens* present in the intestines of chickens in the current study would incite NE [211]. However, as NetB positive and NetB negative bacteria exhibit similar isolation and colonization patterns in chickens [212], it is expected that NetB positive isolates would respond similarly to NetB negative isolates under conditions of physiological stress. This warrants investigation in future studies.

Predictive functional analysis can provide potential insights into bacterial function. In general, metabolic pathways related to carbohydrate biosynthesis and degradation were predicted to be enriched in the small intestine of birds administered CORT. Conversely, amino acid biosynthesis

pathways were predicted to be enriched in the small intestine of control birds. The disproportionately high abundance of *C. perfringens* in CORT-treated birds may account for why some predictive functions were altered between control and CORT treatment birds. For example, *C. perfringens* harbors several carbohydrate-active enzymes that can function to degrade glycans of intestinal mucus [205]. Furthermore, a virulent *C. perfringens* strain (i.e., CP1) has been shown to utilize sialic acid of chicken intestinal mucus and may be a contributing function in the development of NE in poultry [125]. A high protein diet has been proposed as a predisposing factor to *C. perfringens* overgrowth, as the bacterium lacks genes for several amino acid synthesis pathways [213, 214]. The utilization of dietary protein by *C. perfringens* may reduce the host's capacity to assimilate amino acids in an overgrowth state of *C. perfringens,* and potentially render fewer resources for weight gain. I have previously observed impaired weight gain in birds administered CORT at the doses used in the current study, and also observed reduced weight gain in the current study (Appendix C, Figure S2.2) [36]. The relationship among intestinal communities, bird metabolism, and health in the context of bacterial metabolism warrants study. Future research could include prescribed metabolite measures or metabolomics in birds challenged with *C. perfringens* ± stress to provide insights into bacterial functions and potential benefits or consequences to host health.

In conclusion, controlled physiological stress mediated by CORT administration altered bacterial communities in the small intestine, including an increase in the density of *C. perfringens.* Predictive functional analysis identified possible modulations to bacterial function following CORT administration. Given that stress can modulate a variety of host functions, including metabolism, future studies should implement a multi-omics approach to better understand the interactions between the host and the microbiota during their development in chickens, and importantly, examine how this relationship evolves over time under conditions of physiological stress. As birds were not exposed to any antimicrobials in the current study (i.e., as a confounding effect), study findings implicated physiological stress as an important mediator of the microbiota, including *C. perfringens*, and supported stress as a predisposing factor to NE. Future research should include challenging birds with both stress and a known virulent strain of *C. perfringens* to ascertain the mechanisms by which stress predisposes birds to NE. Lastly, deciphering interactions between hosts ± stress and the microbiota will be beneficial to developing novel, non-antibiotic, and tailored strategies in poultry production.

3.6 Figures

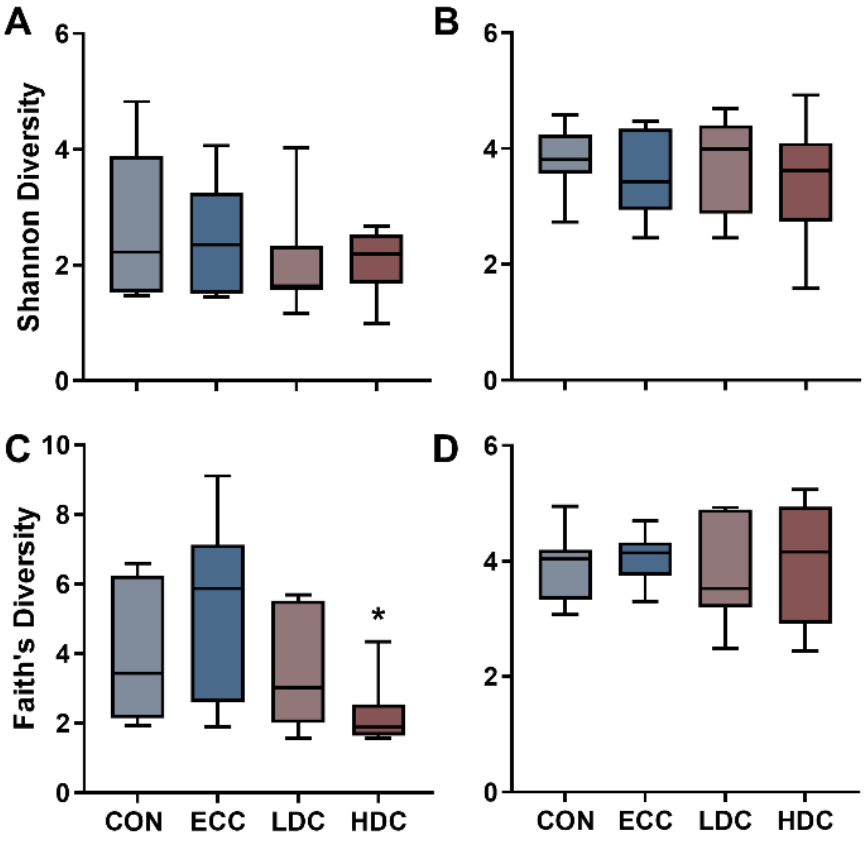

Figure 3.1 Effect of corticosterone administration on α-diversity of bacteria in the small intestine and ceca of chickens. Birds were administered normal drinking water (CON), 0.2% ethanol drinking water (ECC), 10 mg/L CORT (LDC), or 30 mg/L CORT (HDC). Shannon's diversity in the (A) small intestine and (B) ceca. Faith's phylogenetic diversity of the (C) small intestine and (D) ceca. * Indicates $P < 0.050$ in comparison to CON and ECC treatments.

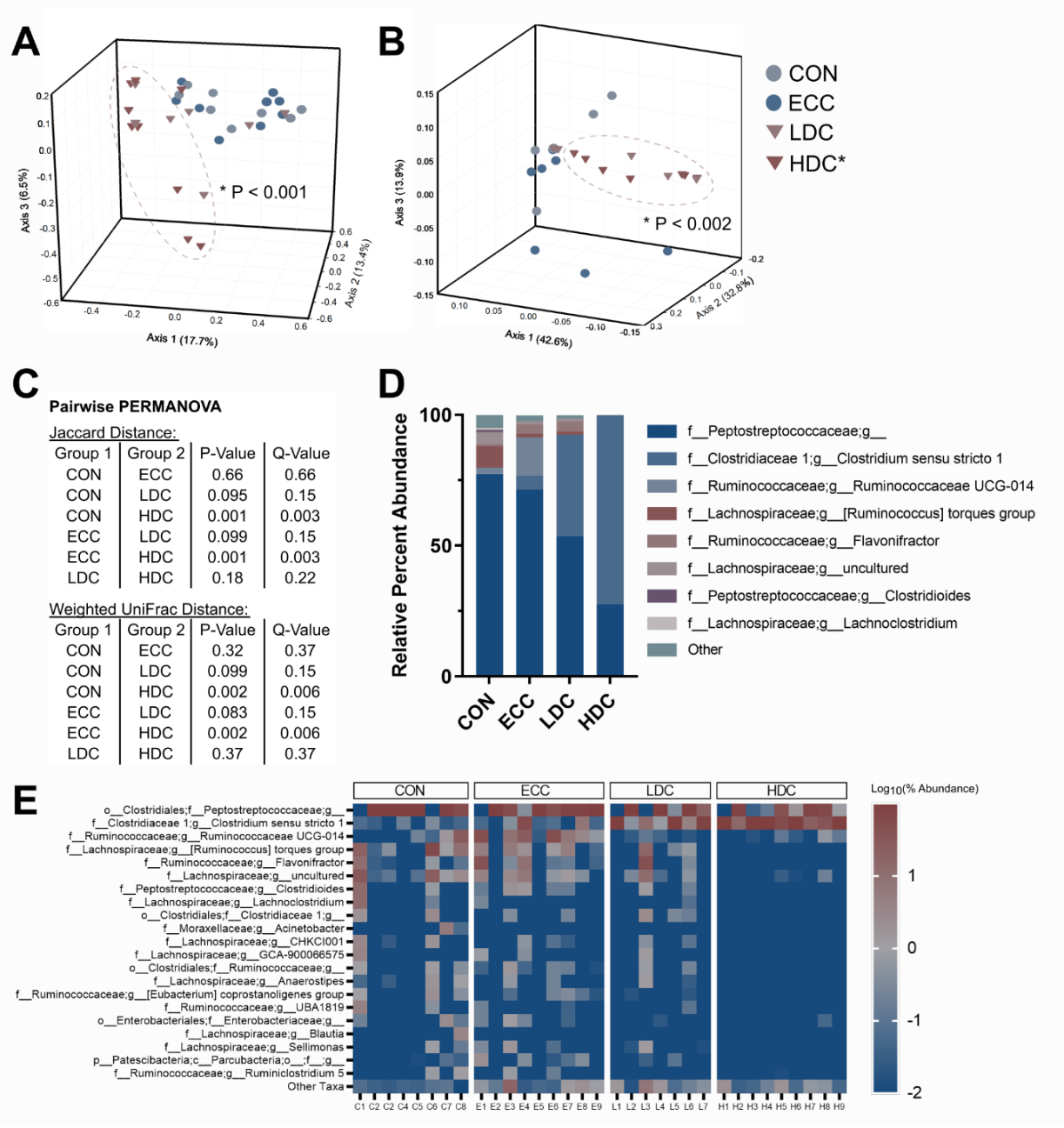

Figure 3.2 Corticosterone administration alters bacterial composition in the small intestine of chickens. Birds were administered normal drinking water (CON), 0.2% ethanol drinking water (ECC), 10 mg/L CORT (LDC), or 30 mg/L CORT (HDC). (A) Jaccard distance and (B) weighted UniFrac PCoA plots of the small intestine. Ellipsoids cluster around HDC treatment. (C) Pairwise PERMANOVA results for Jaccard and weighted UniFrac distance. (D) Percent abundance of the eight most abundant taxa in the small intestine. (E) Heatmap of top twenty-one taxa in the small intestine. n = 8 for CON, n = 9 for ECC and HDC, n = 7 for the LDC treatment. * Indicates that the HDC differed (P ≤ 0.002) from the CON and ECC treatments.

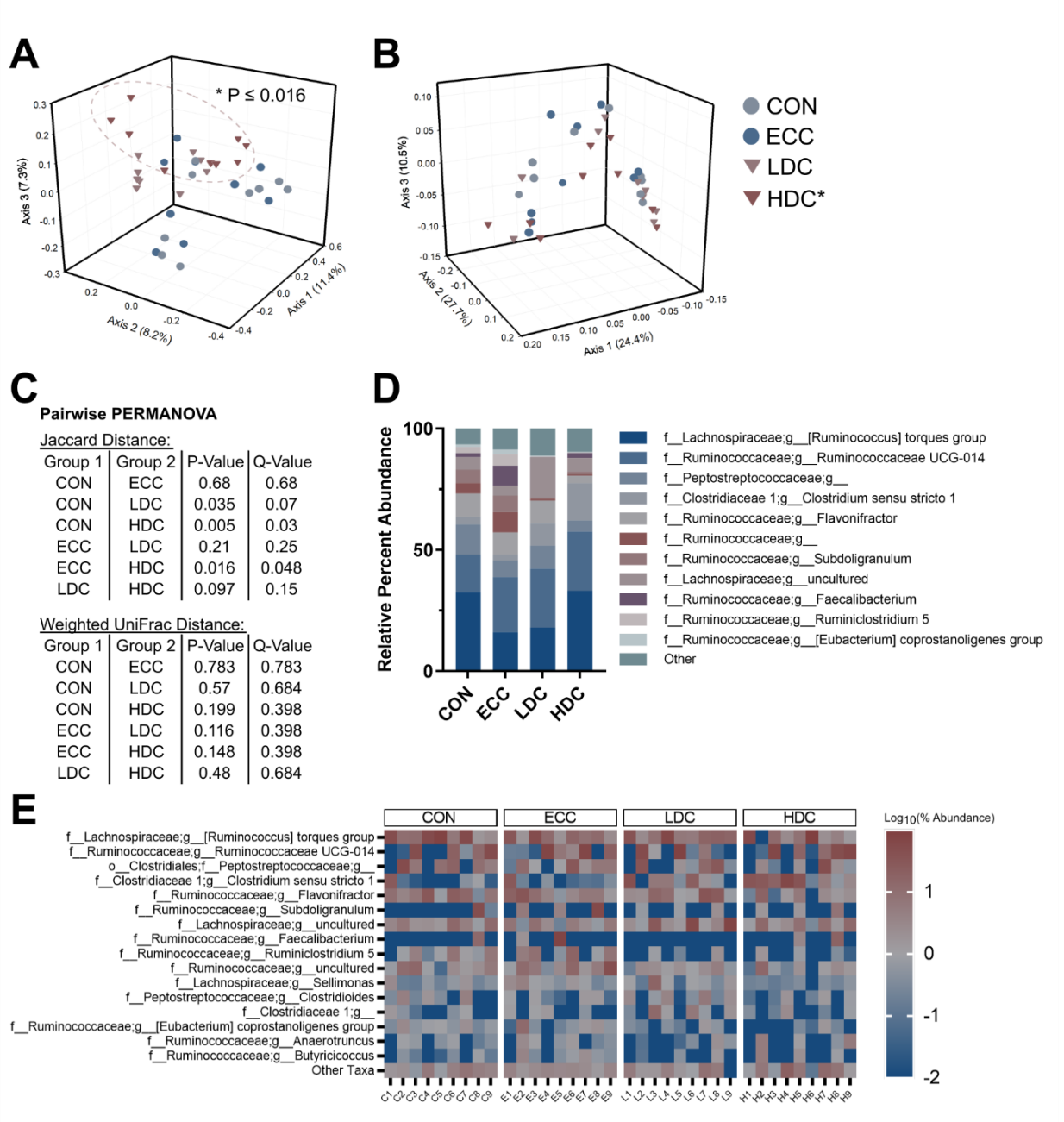

Figure 3.3 High dose corticosterone qualitatively modulates the bacterial communities in ceca of chickens. Birds were administered normal drinking water (CON), 0.2% ethanol drinking water (ECC), 10 mg/L CORT (LDC), or 30 mg/L CORT (HDC). (A) Jaccard distance and (B) weighted UniFrac PCoA plots of cecal bacterial communities. Ellipsoids cluster around HDC treatment. (C) Pairwise PERMANOVA results for Jaccard and weighted UniFrac distance. (D) Percent abundance of top eleven most abundant taxa in ceca. (E) Heatmap of top sixteen taxa in ceca. n = 9 for all treatments. * Indicates that the HDC differed (P ≤ 0.016) from the CON and ECC treatments.

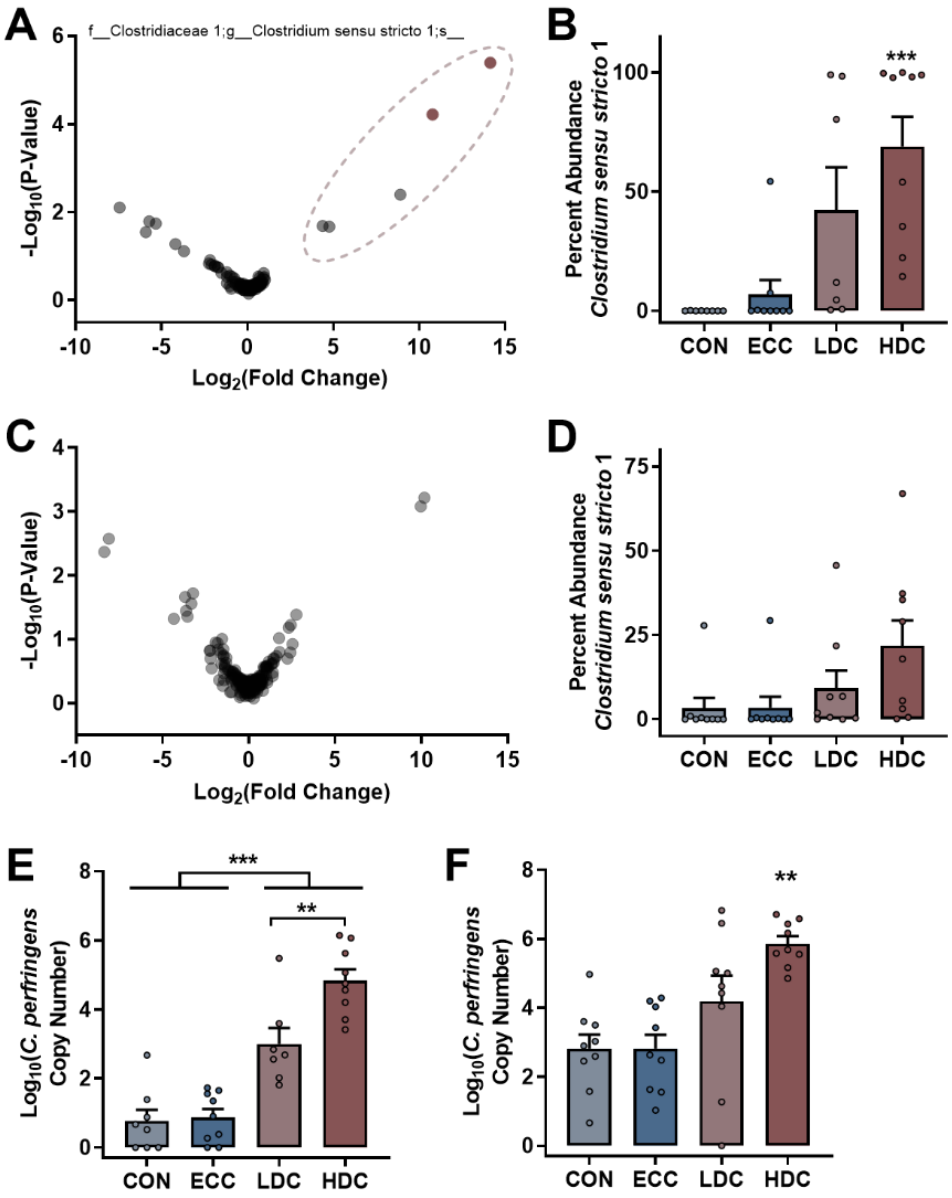

Figure 3.4 ALDEx2 analysis and qPCR determine that *C. perfringens* densities increase in the small intestine and ceca of chickens administered CORT treatment. Birds were administered normal drinking water (CON), 0.2% ethanol drinking water (ECC), 10 mg/L CORT (LDC), or 30 mg/L CORT (HDC). (A and C) Volcano plot constructed from the ALDEx2 output of (A) small intestine and (C) ceca. ASVs within the ellipsoid were identified as *Clostridium sensu stricto 1*, and significant ASVs are shown in pale red. (B and D) Percent abundance of *Clostridium sensu stricto* 1 sequences in the (B) small intestine and (D) ceca. (E–F) qPCR analysis of *Clostridium perfringens* in the (E) small intestine and (F) ceca. ** $P < 0.010$, and *** $P < 0.001$ in comparison to the CON and ECC treatments.

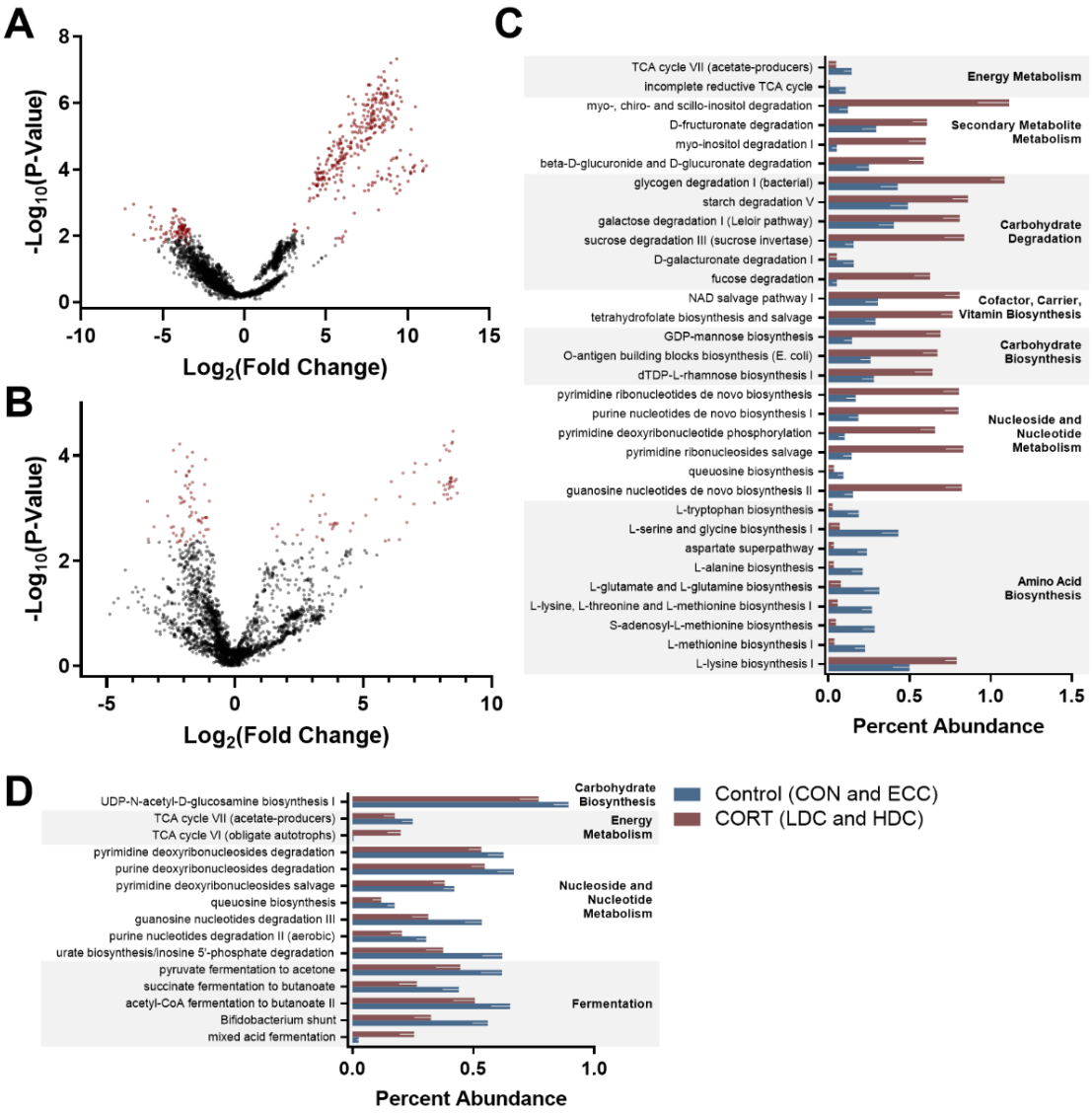

Figure 3.5 ANOVA-Like Differential Expression 2 (ALDEx2) analysis of Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2 (PICRUSt2) output with a MetaCyc pathway abundance in the small intestine and ceca of chickens. Birds were administered normal drinking water (CON), 0.2% ethanol drinking water (ECC), 10 mg/L CORT (LDC), or 30 mg/L CORT (HDC). (A and B) Volcano plot constructed from the ALDEx2 output of (A) small intestine and (B) ceca. Significant Kyoto Encyclopedia of Genes and Genomes (KEGG) ortholog metagenome predictions are shown in red. (C and D) Percent abundance of MetaCyc pathway predictions determined as significant by ALDEx2 analysis for (C) small intestine and (D) ceca.

Chapter 4: Host responses to *Clostridium perfringens* challenge in a chicken model of chronic stress[3]

4.1 Abstract

This study utilized a chicken model of chronic physiological stress mediated by corticosterone (CORT) administration to ascertain how various host metrics are altered upon challenge with *Clostridium perfringens*. Necrotic enteritis (NE) is a disease of the small intestine of chickens incited by *C. perfringens*, which can result in elevated morbidity and mortality. The objective of the current study was to investigate how physiological stress alters host responses and predisposes birds to subclinical NE. Birds administered CORT exhibited higher densities of *C. perfringens* in their intestine, and this corresponded to altered production of intestinal mucus. Characterization of mucus showed that *C. perfringens* treatment altered the relative abundance of five glycans. Birds inoculated with *C. perfringens* did not exhibit evidence of acute morbidity. However, histopathologic changes were observed in the small intestine of infected birds. Birds administered CORT showed altered gene expression of tight junction proteins (i.e. *CLD3* and *CLD5*) and toll-like receptors (i.e. *TLR2* and *TLR15*) in the small intestine. Moreover, birds administered CORT exhibited increased expression of *IL2* and *G-CSF* in the spleen, and *IL1β*, *IL2*, *IL18*, *IFNγ*, and *IL6* in the thymus. Body weight gain was impaired only in birds that were administered CORT and challenged with *C. perfringens*. To conclude, CORT administration modulated a number of host functions, which corresponded to increased densities of *C. perfringens* in the small intestine and weight gain impairment in chickens. Importantly, results implicate physiological stress as an important predisposing factor to NE, which emphasizes the importance of managing stress to optimize chicken health.

4.2 Introduction

Poultry are exposed to many stressors throughout production, which can have costly impacts to producers. Necrotic enteritis (NE) incited by *Clostridium perfringens* is an economically-important disease of the small intestine of poultry that results in high bird mortality and costs the global poultry industry US$5-6 billion per year [1]. Research is unravelling the complex nature that physiological stress imparts on disease development, and stressors can both predispose birds to NE and influence the progression of disease [130, 131, 215]. However, the mechanisms of predisposition are not well understood.

[3] A version of this chapter has been previously published: Zaytsoff, S.J.M., Lyons, S.M., Garner, A.M., Uwiera R.R.E., Zandberg W.F., Abbott D.W., Inglis G.D. Host responses to *Clostridium perfringens* challenge in a chicken model of chronic stress. *Gut Pathog* **12,** 24 (2020).

A number of factors common in poultry production may be involved in predisposition of birds to NE. For example, co-infection with *Eimeria* spp. predisposes birds to NE by promoting epithelial damage and increasing mucus production, which provides nutrient sources that *C. perfringens* can competitively utilize [216, 217]. Dietary factors, such as inclusion of fishmeal and wheat/barley in diets, may also be important predisposing factors for disease [216]. Fishmeal has been demonstrated to alter the composition of the microbiota and may provide novel nutrient substrates for *C. perfringens* growth [218]. Wheat and barley are a source of non-starch polysaccharides, which can increase the viscosity of digesta, increase water intake, and result in wet litter [216, 219]. Limited research has examined how stress affects the physiology of birds, and how this impacts *C. perfringens*, and the initiation and progression of NE.

Stress can promote disease via direct and indirect interactions with pathogens. Studies have demonstrated that neurochemicals produced by the host can interact directly with a bacterial pathogen and influence its growth rate and virulence [220]. This has specifically been shown to occur with *Escherichia coli* O157:H7 and *Vibrio parahaemolyticus* where the catecholamine noradrenaline enhanced virulence properties, such as adherence to the intestinal mucosa and increased expression of the type III secretion system [220]. Physiological stress can indirectly promote disease by altering factors within the intestinal environment and modulate immune function. Stress studies in rats have shown that anxiety- and depression-like behaviour increased goblet cell numbers in the intestine [85]. Likewise, in chickens it has been demonstrated that feed withdrawal increased mucin gene expression in the small intestine [221]. Barrier function is another factor that can be altered during physiological stress [222]. For example, early weaning stress and heat stress in pigs has demonstrated reduced transepithelial electrical resistance in the small intestine [223, 224]. Indirect measures of barrier function in chickens have shown that heat stress can alter the expression of tight junction proteins [93]. Additionally, increased bacterial detection in the spleen can occur in birds challenged with *C. perfringens* [217]. Moreover, physiological stress is known to impact immune function in chickens. In this regard, acute stress has been shown to enhance inflammatory responses, and chronic stress has resulted in immunosuppression [4]. Repeated stress is particularly important to avoid in production as it results in elevated plasma corticosterone (CORT) levels, promotes immunosuppression through disrupting the Th_1-Th_2/T_{reg} balance, and thereby decreases resistance to disease [4].

Modulations to the composition of the enteric microbiota, physical alterations to the gastrointestinal tract, and changes to the immune status of birds are all potential predisposing states to NE, which can be induced by physiological stress. In the current study, white leghorn chickens were

challenged with *C. perfringens* and administered CORT in their drinking water as a method to mediate physiological stress. It is noteworthy that various production stressors (i.e. thermal, social, and ammonia) stimulate the production of CORT in chickens [25, 225, 226]. However, production stressors are inherently variable. Therefore, I chose to exogenously administer CORT to birds to achieve consistently elevated levels of CORT [154]. This allows for the elucidation of how physiological stress effects the host in a prescribed manner. I contend that this will provide crucial baseline information that will facilitate studies to ascertain the impacts of production stressors on predisposition of birds to disease. Notably, the exogenously administered CORT model is well established, and it has been used previously to study alterations to host metrics in chickens [140, 154, 227]. Using the CORT administration model, a primary goal of the study was to induce a subclinical state of NE to ascertain how extended stress can influence host responses and bird growth. I hypothesize that physiological stress predisposes birds to subclinical NE by promoting the proliferation of *C. perfringens and* modulating the host immune system leading to reduced production performance (e.g. weight gain). Objectives of the study were to determine the impacts of CORT administration on: (i) densities of *C. perfringens* in the intestine; (ii) intestinal mucin production and glycan structure; (iii) tight junction proteins and TLR expression; (iv) disease progression (i.e. histopathologic changes and modulated immune responses); and (v) weight gain in birds. Birds were assigned to one of four following treatments: (1) a negative *C. perfringens* (Cp-) and negative stress (St-) treatment (Cp-St-); (2) a positive *C. perfringens* and negative stress treatment (Cp+St-); (3) a negative *C. perfringens* and a positive stress treatment (Cp-St+); or (4) a positive *C. perfringens* and positive stress treatment (Cp+St+).

4.3 Materials and Methods

4.3.1 Ethics statement

The study was carried out in strict accordance with the recommendations specified in the Canadian Council on Animal Care Guidelines. The project was reviewed and approved by the Lethbridge Research and Development Centre (LeRDC) Animal Care Committee (Animal Use Protocol Review #1707) before commencement of the research.

4.3.2 Experimental design

The experiment was designed as a factorial experiment with two levels of *C. perfringens* challenge (±) and two levels of stress (±) arranged as a completely randomized design with four replicates (n=16). The experiment was conducted on two separate occasion (i.e. 'runs'), with two replicates per run. The four treatments were: (1) a negative *C. perfringens* and negative stress treatment (Cp-St-); (2) a positive

C. perfringens and negative stress treatment (Cp+St-); (3) a negative *C. perfringens* and a positive stress treatment (Cp-St+); and (4) a positive *C. perfringens* and positive stress treatment (Cp+St+).

4.3.3 Animals

Specific-pathogen-free white leghorn chickens eggs were obtained from the Canadian Food Inspection Agency (Ottawa, Canada). Eggs were incubated in a Brinsea Octagon 40 Advanced Digital Egg Incubator (Brinsea Products Inc., Titusville, FL) according to the manufacturer's guidelines from incubating chicken eggs. Eggs were maintained at 37.5°C and 60% humidity with hourly turning of the eggs for the first 18 days of incubation. Thereafter, eggs were set flat for hatching and humidity was increased to 70%. Chicks (1-day-old) were placed in pairs within individually ventilated cages (1862 cm^2 floor space; Techniplast, Montreal, QC). These cages were operated in containment mode (i.e. negative air pressure flow) to provide bi-directional HEPA filtered air exchange and protect researchers from pathogens, including *C. perfringens*. Autoclaved wood shavings (United Farmers of Alberta Co-operative Ltd., Lethbridge, AB) were added to each cage, and were replaced each morning. Birds were provided continuous free access to a non-medicated commercial starter diet (Hi-Pro Feeds, Lethbridge, AB; Appendix C, Table S3.1) and water by nipple drinker. Birds were maintained at 30˚C for 2 days, 28˚C for 2 days, then maintained at 26˚C for the remained of the experiment on a 16 hr light: 8 hr dark cycle. Birds were weighed daily starting at 10 days post-hatch.

4.3.4 Corticosterone administration

The dose and method of CORT administration was determined in a previous study [36]. Corticosterone (20 mg; Sigma Aldrich Inc.) was dissolved in 2 mL of anhydrous ethanol and added to 1 L of drinking water (0.2%). Water containing CORT was prepared fresh each day, and added to animal cages twice daily. Corticosterone control birds were administered water containing 0.2% ethanol. Corticosterone and/or ethanol was added to water when chicks reached 14-days-of-age and continued until the end of the experiment.

4.3.5 *Clostridium perfringens* inoculation

A pathogenic strain of *C. perfringens* (CP1) was grown in Heart Infusion Broth in an anaerobic atmosphere using a gas pack (Oxioid™, AnaeroGen™) for 16 hr at 37˚C. At 14-days-of-age, birds were gavaged with 500 µL of *C. perfringens* (5 x 10^7 CFU total) culture grown for 16 hr or Hearth Infusion Broth (control) for 2 consecutive days. To enumerate cell densities, the broth culture was diluted in a 10-fold dilution series, 200 µL was spread onto Columbia agar containing 5% sheep blood, and colonies were counted at the dilution yielding 30 to 300 CFU. Fecal samples were collected 24 hr post-inoculation to confirm infection by the CP1 strain, which is positive for the NetB toxin. Fecal DNA was extracted

using Qiamp Fast Stool Kit (Qiagen, Inc.) and subjected to conventional PCR using primers specific to NetB toxin gene (Table S1).

4.3.6 Bird euthanasia and sample collection

Fecal samples were collected 24 hr post-inoculation with *C. perfringens* using sterile forceps during daily cage cleaning. At the experimental endpoint, all birds were anesthetized, euthanized, and sampled. Birds were anesthetized with isoflurane (5% isoflurane; 1 L O_2/min) and blood was collected via intracardiac puncture. Birds were euthanatized by cervical dislocation while under general anaesthesia. The abdomen was opened with a ventral midline incision, and the thymus, spleen, duodenum, jejunum, and the ceca were aseptically removed. The intestine was longitudinally opened using a sterile blade, and digesta in the lumen of the jejunum and ceca was removed using a sterile wooden splint. Mucus in the duodenum was gently scraped from mucosa using a sterile glass microscope slide. Tissue samples for RNA analysis were immediately placed within RNAlater® (Qiagen Inc., Toronto, ON). Tissues for histopathologic examination were placed in 10% neutral buffered formalin (i.e. for hematoxylin and eosin (H&E) staining). Intestinal tissues for staining and visualization of mucins were placed in methacarn (60% methanol; 30% chloroform; 10% glacial acetic acid). With the exception of samples for H&E and mucin examination via microscopy, samples were stored at -80°C until processed.

4.3.7 Quantitative PCR of *Clostridium perfringens*

Bacterial genomic DNA from duodenal mucus, jejunum digesta, and cecal digesta was extracted using Qiamp DNA Fast Stool Kit (Qiagen, Inc.). Genomic DNA was extracted from a pure culture of *C. perfringens* CP1 strain using DNeasy Blood and Tissue Kit (Qiagen, Inc.). Cell biomass was lysed using enzymatic lysis buffer (20 mM Tris·Cl, pH 8.0; 2 mM sodium EDTA; 1.2% Triton X-100; 20 mg/mL lysozyme) instead of the lysis solution (buffer ATL) supplied in the kit. A standard curve of know copies of 16S DNA specific to *C. perfringens* was generated with DNA amplified from the CP1 strain (primers in Table S1). Amplicons were visualized in a 2% agarose gel, and the amplicon was extracted using QIAquick Gel Extraction Kit (Qiagen Inc.). To generate a standard curve of known gene copies, the gel-extracted DNA was quantified fluorometrically using Qubit™ 2 (Life Technologies, Burlington, ON, Canada), and copies of genes were normalized to 10^7 copies/µL based on concentration, amplicon size, and nucleotide weight. A standard curve was generated by diluting DNA in a 10-fold dilution series and amplifying *C. perfringens* 16S DNA using CP1.2 primers (Table S2). Quantitative PCR (qPCR) was used to measure *C. perfringens* densities in duodenal mucus, jejunum digesta, and cecal digesta relative to the standard curve and normalized by the weight of the sample. Each reaction contained 10.0 µL Quantitect SYBR green master mix (Qiagen Inc.), 1.0 µL of each primer (10 µM), 2.0 µL bovine serum albumin (1

mg/mL), 4.0 µL DNase-free water, and 2.0 µL template DNA. Reactions conditions were: 95˚C for 15 min; and 40 cycles of 95˚C for 15 s, 55˚C for 30 s, and 72˚C for 30 s; and melt curve analysis from 55-95˚C. A MxPro 3005P thermocycler (Agilent Technologies, Santa Clara, CA) was used to conduct qPCR analysis. Reactions were run in triplicate, and mean of the three observations was calculated.

4.3.8 Alcian blue periodic acid Schiff stain and ImageJ quantification

Duodenum and jejunum tissues were fixed in methacarn for a minimum of 48 h. Samples were dehydrated using a Leica tissue processor (Leica TP1020 Benchtop Tissue Processor, Leica Biosystems, Concord, ON), embedded in paraffin blocks using a Shandon Histocentre 3 Embedding Center (Thermo Scientific, Ottawa, ON), sectioned (≈5 µm) using a Finesse 325 Manual Rotary Microtome (Thermo Scientific). Slides were deparaffinised with xylene and rehydrated to water through a series of decreasing ethanol washes. Slides stained with 1% alcian blue (pH 2.5, 3% acetic acid) for 30 min and subsequently rinsed with water for 8 min. Slides were immersed into 0.5% periodic acid for 10 min followed by 5 min washing with water, stained with Schiff solution for 20 min, and rinsed with warm tap water for 10 min. Slides were then dehydrated with 100% ethanol, cleared with xylene and cover slipped with Permount™ mounting medium (Fischer Chemical SP15500). Each tissue sample was visualized with a Zeiss Axioskop2 Plus microscope, and photographed using an Axiocam 506 color camera with Zen 2.0 software. The surface area of alcian blue staining was quantified in eight arbitrarily-selected intact villi and crypts for each tissue section. To ensure uniformity in staining and analysis, all slides were stained and photographed at the same time. The individual completing quantification analysis was blinded to treatments. Alcian blue staining was quantified using ImageJ as previously described [217, 228]. Each picture was imported into ImageJ and made into a RGB stack. The red stack was used for quantification where lower threshold was set to zero and upper threshold set to140. The region of interest tool was used to select either the villi or crypt region. The measure function was used to obtain percent surface area of alcian blue staining in the region of interest.

4.3.9 Characterization of mucus

4.3.9.1 O-glycans. Mucus was transferred to pre-weighed 1.5 mL screw-capped centrifuge tubes (Sarstedt, Germany), dried by lyophilisation (reweighed), and subjected to ammonia-catalyzed β-elimination as previously described [229] with several modifications. In brief, samples were heated in $(NH_4)_2CO_3$ (Sigma) saturated NH_4OH (Anachemia) for 40 hr at 60˚C. After cooling, samples were evaporated to dryness at ambient temperature using a SpeedVac (Thermo) concentrator; 1 mL of 18 $M\Omega cm^{-1}$ water was added to the dried samples which were mixed by vortexing and sonication in a water bath sonicator (VWR), and dried a second time to remove ammonium salts. Samples were suspended in

100 µL of 18 MΩcm^{-1} water and insoluble proteins were pelleted by centrifugation (10,000 x g, 5 min, 21˚C). Without prior acidification or reduction, the released *O*-glycans contained in the supernatant were subsequently desalted using 250 mg Supelco ENVICarb solid phase extraction (SPE) cartridges (Sigma) [125]. Desalted *O*-glycans were labelled with 8-aminopyrene-1,3,6-trisulfonate (APTS) and subsequently resolved by capillary electrophoresis (CE; ProteomeLab PA800; Beckman-Coulter); APTS-labelled glycans were detected by laser-induced fluorescence (LIF) [230, 231]. Peaks were manually integrated using 32 Karat Software Version 7.0 (Beckman-Coulter); in several instances, peaks migrated too closely together to enable accurate comparisons/integrations between different samples, and, in these cases, whole regions of the electropherograms were integrated. All glycan peak areas were expressed relative to the sum of the total integrated areas of peaks not attributable to the APTS labelling reagent. APTS-labelled *O*-glycans were treated with bovine kidney α-fucosidase (Sigma) or mild acid hydrolysis (see below) in order to identify fucose- and sialic acid-containing glycans, respectively; in each case, loss of a CE peak (relative to a non-treated control) after treatment was taken as evidence for these monosaccharide constituents.

4.3.9.2 Sialic acid quantitation. Lyophilized mucus samples were accurately weighed, suspended in 2 M acetic acid (EMD Millipore), and subjected to mild acid hydrolysis to selectively liberate sialic acids exactly as previously described [232]. The released sialic acids were derivatized using 1,2-diamino-4,5-dimethylbenzene (DMBA; Sigma) and quantitated by high-performance liquid chromatography-mass spectrometry (HPLC-MS) using an external calibration curve. The amount of sialic acid present in each sample was either normalized to the dry sample mass or to the total monosaccharide content as determined by the phenol-sulfuric method [233].

4.3.10 Quantification of mRNA of response genes

Total RNA was extracted from duodenum, jejunum, spleen, and thymus tissues using a RNeasy mini kit (Qiagen Inc.) as described previously [36]. An additional DNase step was included to remove residual genomic DNA. A Bioanalyzer RNA 6000 Nano kit (Agilent, Mississauga, ON, Canada) was used to measure RNA quality and quantity, and 1 µg of RNA was reverse transcribed to cDNA using a QuantiTect reverse transcription kit (Qiagen Inc.). Quantitative PCR was performed using an MxPro 3005P thermocycler (Agilent Technologies). Each reaction contained 5.0 µL of Quantitect SYBR green master mix (Qiagen Inc.), 0.5 µL of each primer (10 µM), 3.0 µL RNase-free water, and 1.0 µL cDNA. PCR conditions were: 95°C for 15 min; 40 cycles of 95°C for 15 sec, 58°C for 30 sec, and 72°C for 30 sec; and melt curve analysis from 55-95°C. Primer sequences specific to gene targets (Appendix C, Table S3.2) were generated using NCBI primer BLAST; primers were designed to create an amplicon between 75 and 200

base pairs. Efficiencies for all primers were between 95–110% and a single peak was present in melt curve analysis. Reactions were run in triplicate and the average ct values were used to calculate gene expression relative to two reference genes (*BA* and *TBP)* using qBase+ software (Biogazelle, Gent, Belgium) [158].

4.3.11 Small intestine histopathology

Jejunum tissue samples were fixed in formalin for minimum 24 hr. Samples were dehydrated, embedded in paraffin, and sectioned as described above. Slides were de-paraffinized with xylene and stained with hematoxylin and eosin. Jejunal sections were scored by veterinarian pathologist (R.R.E.U) blinded to treatments using modified scoring criteria previously described [234, 235]. Sections were graded 0 to 4 for villus fusion, villous hemorrhage, epithelial cell injury, red blood cells within lumen, proteinaceous material within the lumen, intestinal inflammatory infiltrates, and fibrosis. Total pathological score was determine by calculating the sum of scores from all categories for each bird.

4.3.12 Statistical analysis

Statistical Analysis Software (SAS Institute Inc. Cary, NC) was used to perform the majority of statistical analysis except as otherwise noted. With the exception of the histopathologic score data, continuous data was assessed to ensure normality. Treatment and interaction among factors were determined using a mixed linear model. Animal body weight data was treated as a repeated measure; the appropriate covariance structure was utilized according to the lowest Akaike's Information Criterion. In the event of a significant effect ($P \leq 0.050$), the least squares means test was used to evaluate differences among treatments for qPCR, gene expression, mucin stain intensity, and body weight gain. Categorical data (i.e. histopathologic changes) was analyzed using a non-parametric Fisher's exact test where pairwise comparisons were performed between all treatments. Differences in glycan abundances were analyzed using R (Version 3.6.1). The normality and homogeneity of variances in the average relative (%) abundances of each *O*-glycan was assessed using normal quantile plots and a Levene's test, respectively. An analysis of variance (ANOVA) was subsequently employed to determine if there was a difference (at a significance level of 0.05) in *O*-glycan levels associated with each bird treatment. A Tukey's honestly significant difference (HSD) test was performed to determine statistically significant differences between the means of each group with a significance level of 95 % ($P < 0.050$). A Spearman's rank correlation test was performed (using Microsoft Excel) to discover co-varying *O*-glycans. Data are represented as the mean ± standard error of the mean.

4.4 Results

4.4.1 Corticosterone treatment was associated with increased densities of *Clostridium perfringens* in the small intestine

Confirmation of the successful colonization of inoculated birds by CP1 *C. perfringens* was confirmed by conventional PCR for the NetB toxin gene, and showed that only inoculated birds (Cp+St- and Cp+St+) were positive for *netB* 24 hr post-inoculation (Appendix C, Figure S3.1). Moreover, birds administered CORT (Cp-St+ and Cp+St+) showed increased densities of *C. perfringens* in duodenal mucus ($P = 0.035$), jejunal mucus ($P = 0.033$), and jejunal digesta ($P = 0.050$) in comparison to birds not administered CORT (Cp-St- and Cp+St-; Figure 4.1).

4.4.2 Corticosterone affected mucus production in the small intestine

Duodenal and jejunal tissue sections were prepared and stained with alcian blue. The intensity of alcian blue staining was quantified as a percentage of acidic mucins relative to total size of villi or crypt region (Figure 4.2A). Increased mucin staining was observed in the duodenum of birds administered CORT (Cp-St+ and Cp+St+) in both villi ($P = 0.039$) and crypts ($P = 0.012$; Figure 4.2B). No changes ($P = 0.970$) were observed in the jejunum. Relative quantities of *MUC2B* and *MUC5AC* mRNA were measured in the duodenum, and decreased ($P = 0.045$) quantities of *MUC2B* mRNA was observed in birds administered CORT (Cp-St+ and Cp+St+). Quantities of *MUC5AC* mRNA were unaltered ($P \geq 0.670$) by both CORT administration and *C. perfringens* inoculation in the duodenum (Figure 4.2C). Decreased ($P \leq 0.047$) quantities of *MUC5AC* mRNA were observed in the jejunum of Cp-St- treatment birds relative to all other treatments. There was no effect ($P \geq 0.960$) of *C. perfringens* or CORT on quantities of MUC2B mRNA in the jejunum.

4.4.3 Altered mucus glycosylation in *Clostridium perfringens*-infected birds

Total mucin-linked carbohydrates determined using the phenol-sulfuric acid test were 103 ± 25 (Cp-St-), 193 ± 62 (Cp-St+), 78 ± 32 (Cp+St-), and 141 ± 30 (Cp+St+) µmol/mg of mucus. *O*-glycans were chemically liberated from mucins and fluorescently derivatized (Figure 4.3A) to enable rapid glycan profiling by Capillary Electrophoresis with Laser Induced Fluorescence (CE-LIF; Figure 4.3B; Appendix C Figure S3.2). Notably, all glycans share a single common fluorophore, rendering the molar detector response independent of glycan structure, and thus permitted substrate-product relationships to be inferred. In all samples, at least 50 glycans of differing electrophoretic mobilities were detected in under 7 min; peak areas for 23 of these glycans were accurately determined, but areas for three closely migrating glycans (peaks 5, 6, and 19) could not be reliably assigned, and hence larger regions were integrated. Many of the *O*-glycans were inferred to be sulfated, given that only peaks 5 and 7 were

sensitive to weak acid hydrolysis (indicative of the presence of sialic acids), and given that neutral disaccharides migrate around 5 min, and the addition of sulfate groups decreases migration times by *ca.* 1 min. Notably, peaks 5 and 7 (and 22 and 21) were also fucosidase-sensitive, while peaks 17, 18, 22, 23 and 24 conspicuously increased in fluorescence intensity after sialic acid hydrolysis (Appendix C, Figure S3.3). Relative abundances of five *O*-glycans were observed to vary ($P < 0.050$) in birds inoculated with *C. perfringens*; *O*-glycan 3 and 17 both increased ($P < 0.001$), while 7, 10, and 11 decreased ($P < 0.001$; Figure 4.3C), with over a three-fold decrease in glycan 7 (a fucose and sialic acid-containing glycan) being the most pronounced change. The inverse relationship between glycan 7 ($P < 0.001$) and 17 ($P = 0.007$) suggested that these might co-vary. Thus, a Spearman rank correlation test (Figure 4.4A) was performed with all 26 glycans for birds in all treatment groups (n=16). Correlations ($P < 0.001$) among all five glycans that differed ($P < 0.050$) between *C. perfringens*-inoculated (Cp+St- and Cp+St+) and non-inoculated birds (Cp-St- and Cp-St+) were observed. *C. perfringens* can utilize sialic acid as a carbon source *in vitro* [125]. Coupling this knowledge with the observed inverse correlation between glycan 7 and 17 ($P = 0.006$), which were also inversely correlated under desialylation conditions (Figure S3), total sialic acids (Figure 4.4B) were quantitated by HPLC-MS (Figure 4C). No changes ($P > 0.050$) in sialic acid levels were apparent, although three of the four birds inoculated with *C. perfringens* exhibited higher than average sialic acid levels per mg mucus, a trend that was reversed by CORT administration.

4.4.4 Corticosterone affected the duodenal epithelium

Birds administered CORT (Cp-St+ and Cp+St+) exhibited decreased quantities of *TLR2A* ($P < 0.041$) and *TLR15* ($P < 0.047$) mRNA (Figure 4.5A). Neither CORT administration or *C. perfringens* inoculation had an effect ($P \geq 0.970$) on TLR mRNA in the jejunum. Relative quantities of *CLD3* mRNA were higher ($P \leq 0.018$) in the duodenum of birds administered CORT alone (Cp-St+), and highest ($P < 0.001$) in the duodenum of birds inoculated with *C. perfringens* and administered CORT (Cp+St+; Figure 4.5B). In the jejunum, quantities of *CLDN3* mRNA were only higher ($P = 0.011$) in inoculated birds administered CORT (Cp+St+) in comparison to Cp-St- treatment birds. Relative quantities of *CLDN5* mRNA in the duodenum were lower ($P = 0.042$) in birds administered CORT (Cp-St+ and Cp+St+). Neither CORT administration or *C. perfringens* inoculation had an effect ($P \geq 0.110$) on quantities of *OCLN* mRNA in the duodenum or jejunum.

4.4.5 Corticosterone and *Clostridium perfringens* induced histopathologic changes in the small intestine

No macroscopic lesions characteristic of NE were observed in the small intestine of any birds. However, histopathologic changes ($P < 0.029$), albeit it moderate were observed for birds inoculated

with *C. perfringens* (Cp+St- and Cp+St+) in comparison to birds not inoculated with the pathogen (Cp-St- and Cp-St+; Figure 4.6). Notably, villi hemorrhage contributed to higher ($P \leq 0.050$) histopathological scores in Cp+St+ birds in comparison to birds not inoculated with *C. perfringens* (Cp-St- and Cp-St+). Fibrosis was also elevated ($P = 0.037$) in Cp+St+ birds in comparison to Cp-St- birds.

4.4.6 Corticosterone and *Clostridium perfringens* modulated immune responses in the spleen and thymus

Relative quantities of *IL2* ($P = 0.043$) and *G-CSF* ($P = 0.030$) mRNA were higher in the spleen of birds administered CORT (Cp-St+ and Cp+St+; Figure 4.7A). In contrast, CORT administration did not alter ($P \geq 0.190$) mRNA levels of *IL1β*, *IL18*, *IFNγ*, *IL6,* or *TGFβ* in the spleen. In the thymus, relative quantities of *IL1β*, *IL2*, *IL18*, *IFNγ*, *IL6*, and *TGFβ* mRNA were higher ($P \leq 0.005$) in birds administered CORT (Cp-St+ and Cp+St+; Figure 4.7B).

4.4.7 Administration of corticosterone and *Clostridium perfringens* in combination reduced weight gain

Only birds administered *C. perfringens* and CORT (Cp+St+) exhibited reduced ($P \leq 0.050$) weight gain relative to other treatments (Figure 4.8). This effect was initially detected ($P \leq 0.015$) 3 days after inoculation with *C. perfringens* and commencement of CORT administration, and persisted ($P \leq 0.005$) for the remainder of the experimental period.

4.5 Discussion

The mode in which physiological stress predisposes birds to disease is complex in nature and includes a variety of factors that can be modulated within the host. In the current study CORT was administered to birds to determine if physiological stress can modulate host responses within the intestine and immune organs in a manner that could predispose them to NE. Additionally, I investigated the impact of *C. perfringens* and physiological stress on body weight gain and subclinical NE.

4.5.1 *Clostridium perfringens* densities in the small intestine

The risk of NE is elevated when higher densities of *C. perfringens* are present in the intestine of chickens [236]. It has been demonstrated that heat stress, cold stress, and high stocking density in chickens can elevate *C. perfringens* cell densities in ceca [130-132]. In the current study, I examined the impact of stress on *C. perfringens* densities in the small intestine as it is the primary site of NE incitation. Notably, all birds were colonized with non-NetB *C. perfringens* strains that presumably originated from eggs (i.e. the specific-pathogen-free leghorn chickens used in the study are not *C. perfringens* free). However, only birds inoculated with NetB-containing *C. perfringens* were positive for *netB* detection in the feces. Corticosterone administration was associated with an in increase in NetB and non-NetB *C.*

perfringens densities within duodenum mucus, jejunum mucus, and jejunum digesta. Higher intestinal densities of *C. perfringens* result in elevated fecal shedding of *C. perfringens* into the environment which in turn exposes birds to re-inoculation by the bacterium, and horizontal transmission to other birds [132]. Moreover, higher densities of *C. perfringens* may facilitate quorum sensing and increased release of toxins in the intestine [128]. For example, the VirSR two-component system has been shown to transcriptionally regulate the NetB toxin, which is considered to be an important pathogenicity factor in *C. perfringens*. Thus, higher numbers of *C. perfringens* in the intestine would be expected to elevate the probability of NE development.

4.5.2 Alterations to intestinal mucus

Physiological stress can alter the intestinal environment in a manner that provides novel substrates to microorganisms. In this regard, it has been hypothesized that *C. perfringens* can utilize intestinal mucins as a mechanism to gain access to host epithelium [217]. Recent research by collaborators has demonstrated that *C. perfringens* is capable of growing on the mucin monosaccharides D-galactose, D-mannose, and sialic acid (*i.e*. *N*-acetyl-D-neuraminic acid, and *N*-acetyl-D-glucosamine) when provided as a the sole carbon source [125]. Furthermore, a piglet model demonstrated selection for *C. perfringens* under enhanced mucus production [199]. In the current study, intestinal mucins were visually measured by staining with alcian blue, and quantified the intensity of mucin staining within the small intestine. Increased mucin staining in the duodenum in both villi and crypt regions of birds administered CORT was observed. A relationship between *C. perfringens* densities and increased goblet cell numbers has previously been observed in ileal crypts of broiler chickens [217]. The density of *C. perfringens* was highest in birds administered CORT, which corresponded with the increased mucus production detected in the duodenum of birds. Previous reports have demonstrated increased expression of *MUC5AC* in the jejunum of *Eimeria* spp. challenged birds, and decreased expression of *MUC2B* in birds co-challenged with *C. perfringens* and *Eimeria* spp. [237]. Increased *MUC5AC* in the jejunum of birds administered *C. perfringens* alone, CORT alone, and *C. perfringens* and CORT in combination was observed. A decrease in the expression of *MUC2B* was only observed in the duodenum of birds administered CORT. Visual staining of mucins did not correspond with the gene expression results. In this regard, increased intensity of mucin staining within duodenum crypts and villi was observed, but no changes in mucin quantities in the jejunum among treatments was observed. One possibility is that measuring mRNA gene expression may not be an accurate way to assess the levels of mucins present in the intestine. Furthermore, I sampled birds 7 days post-infection, which may have been too late to detect many

changes in mucin gene expression. It is noteworthy that a previous study also reported no change in *MUC2B* expression after a 7 day *C. perfringens* challenge [238].

Intestinal mucins are up to 80 percent O-glycans by mass. Thus, changes in the biosynthesis and/or metabolism of mucin *O*-glycans (i.e. by *C. perfringens* or other intestinal microorganisms) may have resulted in increased levels of acidic- (Neu5Ac- or sulfate-containing) and alcian blue-stained glycans even as mRNA levels for MUCs decrease. A trend for higher glycosylation of mucus in birds administered CORT was observed. This observation corresponded with increased intensity of alcian blue staining in the duodenum. Alterations to O-glycan profiles were further assessed through CE-LIF. A relative decrease in glycans containing sialic acid (glycan 7) and/or fucose (glycan 7 and 10) with a concurrent increase in sulfated glycans (glycans 3 and 17) in birds inoculated with *C. perfringens* was observed. It is generally accepted that the sulfation of intestinal mucins confers resistance to microbial degradation [239, 240]. These results indicate that *C. perfringens* could modify the composition of mucins, although it is unknown whether this occurred due to *C. perfringens* utilization of O-glycans, or because host production of mucins was modified due to infection by *C. perfringens*. Future experiments in the presence of sialidase inhibitors will aid in resolving how *C. perfringens* can alter the O-glycan composition of intestinal mucins [241]. In addition, techniques permitting absolute glycan quantitation (rather than assessing relative levels as was done here and elsewhere [242]) will be needed to fully resolve transcriptional and post-transcriptional alterations in mucin biosynthesis.

4.5.3 Modifications to small intestine epithelium

Epithelial properties were examined by measuring mRNA expression of tight junction proteins and TLRs. Claudin 3 and 5 function to maintain barrier integrity by regulating paracellular permeability to ions, solutes, and proteins [243]. Both *C. perfringens* and CORT administration incited elevated expression of *CLD3* in the duodenum. Therapeutic use of glucocorticoids in humans has been shown to enhance intestinal epithelial tight junction integrity, and this corresponds with increased claudin expression [90]. Conversely, increased expression of *CLD3* in alveolar epithelial cells has been associated with a decrease in barrier function [244]. Toxigenic intestinal pathogens are capable of disrupting barrier function by altering tight junction proteins [245, 246], and given the toxigenic nature of *C. perfringens*, it is possible this influenced the alterations to claudin expression that was observed in the current study. Heat stress in chickens has been shown to increase *CLD5* expression in the jejunum and ileum [93]. Likewise, mycotoxin treatment resulted in an increase in *CLD5*, which may have been the result of subclinical inflammation [247]. In contrast to claudins, occludin was unaffected by *C. perfringens* and/or CORT administration as has been previously reported in birds exposed to heat stress [93]. Occludin is a

tight junction protein that functions in maintaining barrier integrity, although is not always essential for tight junction formation [248].

Toll-like receptor 2 signalling has been implicated in the preservation of intestinal barrier integrity under a state of inflammation [249]. For example, mice with colitis induced by dextran sulfate sodium exhibited ameliorated clinical symptoms and increased intestinal integrity when treated with a *TLR2* ligand [250]. One function of *TLR2* is to activate nuclear factor-κB resulting in the release of inflammatory cytokine and chemokine mediators [249]. Additionally, *TLR2* mediates an anti-inflammatory effect by inducing the release of *IL10* which in turn inhibits macrophage and dendritic cell effector function [249]. In the current study, the expression of *TLR2* and *TLR15* in the small intestine of chickens was measured. *TLR15* is a unique TLR to avian species, which exhibits homology with *TLR2* [251]. Although a specific ligand to TLR15 has not been identified, it has been demonstrated that both Gram positive and Gram negative bacteria can stimulate expression of *TLR15* mRNA [251]. This study showed a similar expression pattern for *TLR2* and *TLR15* consistent with a previous report [252]. This suggests that both of these TLRs are regulated or stimulated in the same manner [252]. The administration of CORT to chickens in the current study resulted in decreased expression of both *TLR2* and *TLR15* in the duodenum. Strenuous exercise at high heat has been shown to decrease the expression of *TLR2* in human peripheral monocytes [253]. Likewise, these results are in agreement with a study that showed heat stress in chickens challenged with *Salmonella enterica* Enteritidis exhibited decreased *TLR2* expression in the spleen and cecal tonsils [92]. Decreased levels of *TLR2* have been associated with impairment of intestinal integrity [92]. Moreover, it has been shown that *TLR2* signalling can preserve the integrity of the tight junction protein, zona occluden 1 under inflammatory stress-induced damage [254]. Given the importance of tight junction proteins and TLRs in the maintenance of barrier function, these results indicate that physiological stress induced by CORT administration can modify tight junction protein and TLR expression in the small intestine.

4.5.4 *Clostridium perfringens* challenge induces subclinical necrotic enteritis

Neither *C. perfringens* alone or in combination with CORT incited macroscopic lesions indicative of NE in the small intestine 7 days post challenge with the bacterium. Lesion scores in broiler chickens are highest 1 day after the administration of *C. perfringens*, and NE lesions become inconspicuous 7 days post challenge [255]. However, this study intentionally used a layer breed of chicken that is less susceptible to NE [115] as the goal of this study was to examine chronic stress on sublethal NE. In addition to examinations for gross pathologic changes, histopathologic changes in the small intestine were examined. Conspicuous, albeit moderate, microscopic tissue injury in birds treated with *C.*

perfringens alone and in combination with CORT was observed. Importantly, *C. perfringens* inoculated birds presented substantially higher total histopathological scores than non-inoculated birds indicative of subclinical NE. These results are in agreement with a study that examined the impact of heat stress on necrotic enteritis. In this regard, Calefi et al. [133] observed that birds challenged with *C. perfringens* exhibited higher histopathological scores, while the application of heat stress decreased histopathological scoring in birds challenged with the pathogen. It is noteworthy that this study also observed a small, yet significant, decrease in histological scoring of birds subjected to both *C. perfringens* and CORT. This may be due to the immunomodulatory functions of CORT, which can interfere with the production of inflammatory cytokines and prevent the infiltration of heterophils to the mucosa [133].

To further assess subclinical disease incited by *C. perfringens*, the expression of several immune cytokines in the thymus and spleen were examined. Responses in a primary and secondary lymphoid organ were also examined, as immune responses in the intestine have been previously investigated in *C. perfringens* challenge and stress studies [132, 217, 256, 257]. The spleen is a secondary lymphoid organ that plays a central role in the establishment of the immune system in young birds [258]. The chicken spleen hosts a multitude of functions, which include immune surveillance, lymphocyte differentiation, antibody synthesis, formation of complement, differentiation of blood monocytes into macrophages, and is a primary site for immune complex formation [63, 258]. As chickens lack defined lymph nodes, the spleen is an important organ for disease resistance [63]. The administration of CORT to birds altered the expression of *IL2* and *G-CSF* in the spleen. Interleukin 2 functions to activate and proliferate T cells and is considered to be mediator of a Th1 response [259]. Although elevated *IL2* was observed, the expression of other inflammatory cytokines (i.e. *IL18*, *IFNγ*, *IL6*) were not altered by CORT administration or *C. perfringens* challenge. Elevated *G-CSF*, also known as colony stimulating factor 3, was observed, which may have been regulating the maturation and function of heterophils as has been suggested previously [260]. The elevated *G-CSF* that was observed could correspond with reports of CORT impacts on birds inducing elevation of heterophil numbers [4]. It is noteworthy that Hong et al. [261] demonstrated elevated *IL1β* and *IL6* in the spleen of broilers as early as 2 days post-infection with *C. perfringens*. Although it cannot be excluded that acute responses occurred in white leghorns, the significant differential expression of immune genes that was observed 7 days after inoculation with *C. perfringens* is consistent with a prolonged sublethal infection.

In contrast to responses in the spleen, substantial modulation of immune genes in the thymus of birds administered CORT was observed. The differing responses observed in these two organs suggest

that glucocorticoids impact these organs differently [262]. The spleen consists primarily of mature immune cells that may be more resistant to the actions of glucocorticoids [262]; whereas, the thymus contains immature thymocytes that may be more sensitive to glucocorticoids [262]. Both the bursa of Fabricius and thymus can be highly affected by glucocorticoids through receptor mediated binding that leads to apoptosis [63]. My research has previously demonstrated that CORT can induce lymphoid cell depletion, atrophy, and elevate *IL6* and *TGFβ* responses in the bursa [36]. Furthermore, the elevated expression of the inflammatory cytokines, *IL2*, *IL18*, *IL1β*, *IFNγ*, and *IL6* observed in the thymus after 7 days of CORT administration. This may primarily be due to the local effect of thymocytes undergoing glucocorticoid-mediated cell death with macrophages actively removing debris [262].

4.5.5 Modulation of body weight gain

Clinical NE is recognized as a problem due to high mortalities and losses to farmers. Subclinical disease can also result in tremendous losses as the impacts of disease can go undetected but adversely affect bird performance (e.g. reduced weight gain). Significantly, an impairment in weight gain can translate into significant production losses, as more resources are required to reach slaughter weight. Although white leghorn chickens inoculated with NetB *C. perfringens* were colonized by the bacterium, which incited modest histopathologic changes to the small intestine, there was no observed impairment in weight gain. This is in agreement with other NE studies that showed *C. perfringens* colonization did not affect the weight of broiler chickens [256]. However, there was significant impairment of weight gain observed in birds infected with *C. perfringens* and administered CORT. Although the precise mechanisms responsible for this observation remain enigmatic, my research has previously shown that CORT administration conspicuously affects bird metabolism [36]. Furthermore, mounting an immune response is metabolically costly and it is plausible that the metabolic cost to birds resulting from physiological stress and infection by *C. perfringens* resulted in the reduction in bird weight gain [263]. This study observed a significant weight gain reduction beginning at 3 days post infection with *C. perfringens* and initiation of CORT administration. An examination of host responses earlier in a time course should be conducted in future studies. Moreover, temporal determinations of the metabolic cost to birds sub-lethally infected with pathogens under conditions of physiological stress is warranted.

4.5.6 Conclusion

To conclude, chronic physiological stress mediated by CORT administration altered several host measures that corresponded with increased susceptibility to NE in chickens. Several alterations to mucus characterization, epithelial properties, and immune measures that corresponded with the administration of CORT. Importantly, CORT administration resulted in increased densities of *C.*

perfringens in the small intestine, and impacted bird weight gain in infected birds. These findings emphasize the importance of controlling stress in production not only to enhance bird welfare and performance, but also to influence host-microorganism interactions and decrease the predisposition of birds to disease, including subclinical manifestation of NE and possibly other diseases of chickens.

4.6 Figures

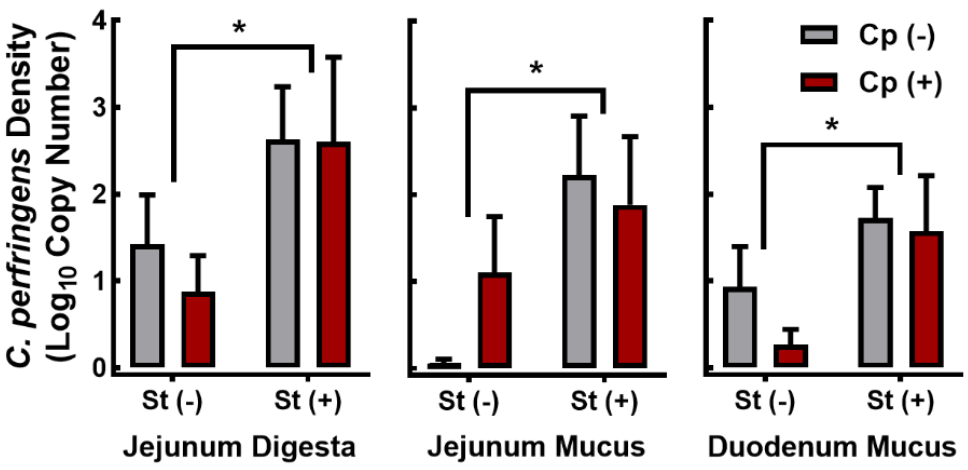

Figure 4.1 Corticosterone-mediated stress increased densities of NetB and non-NetB *C. perfringens* in the small intestine. Birds were administered 0.2% ethanol drinking water (Cp-St-), challenged with 10^7 CFU *C. perfringens* (Cp+St-), administered 20 mg/L CORT (Cp-St+), or received both *C. perfringens* and CORT challenge (Cp+St+). Treatments commenced in birds at 14-days-of-age, where *C. perfringens* was administered for 2 days and CORT for 7 days. Densities of *C. perfringens* (log_{10} copies/g mucus or digesta) were determined by quantitative PCR in jejunum digesta, jejunum mucus, and duodenum mucus using primers specific for 16S gene of *C. perfringens*. Vertical lines associated with histogram bars represent standard error of the means (n = 4). Asterisks indicate significant differences ($P < 0.050$).

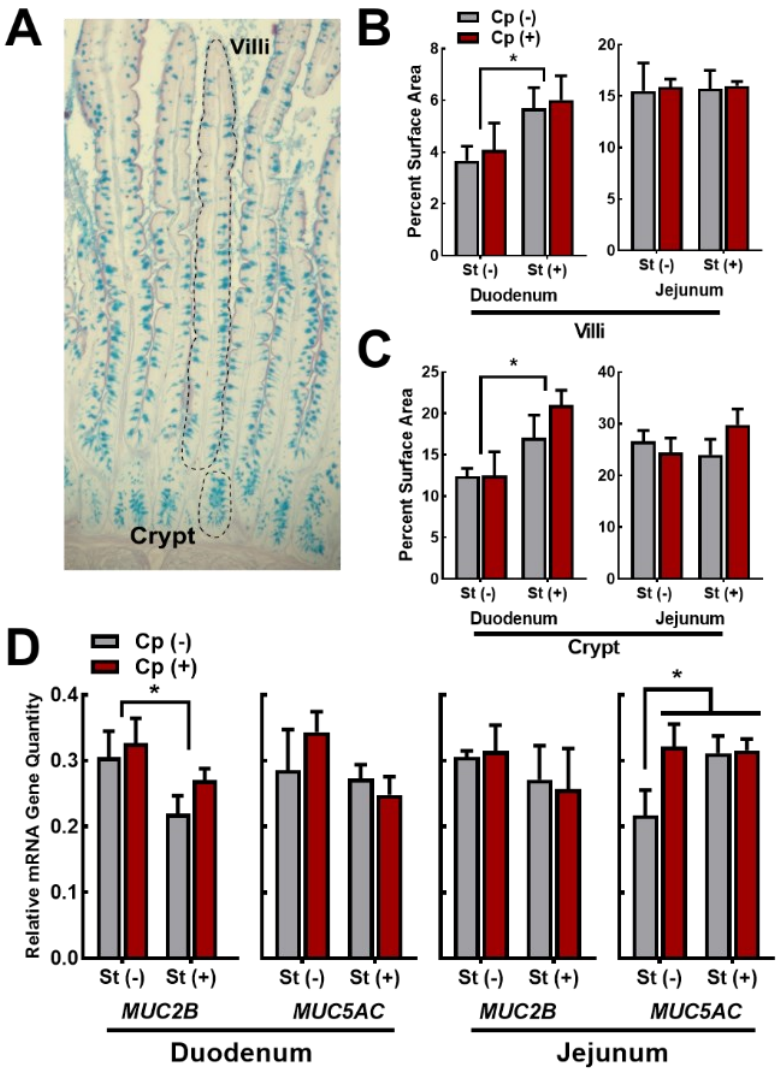

Figure 4.2 *Clostridium perfringens* infection modulated mucus production. Birds were administered 0.2% ethanol drinking water (Cp-St-), challenged with 10^7 CFU *C. perfringens* (Cp+St-), administered 20 mg/L CORT (Cp-St+), or received both *C. perfringens* and CORT challenge (Cp+St+). Treatments commenced in birds at 14-days-of-age, where *C. perfringens* was administered for 2 days and CORT for 7 days. (A) Example of duodenum tissue section stained with alcian blue periodic acid Schiff stain; a individual villus and crypt are indicated with the dotted lines. (B-C) ImageJ quantification of alcian blue staining presented as percent surface area of alcian blue staining to total area of villi or crypt region. (B) Villi alcian blue staining in the duodenum and jejunum. (C) Crypt alcian blue staining in the duodenum and jejunum. (D) Relative mRNA gene quantity of *MUC2B* and *MUC5AC* in duodenum and jejunum. Vertical lines associated with histogram bars represent standard error of the means (n = 4). Asterisks indicate significant differences ($P < 0.050$).

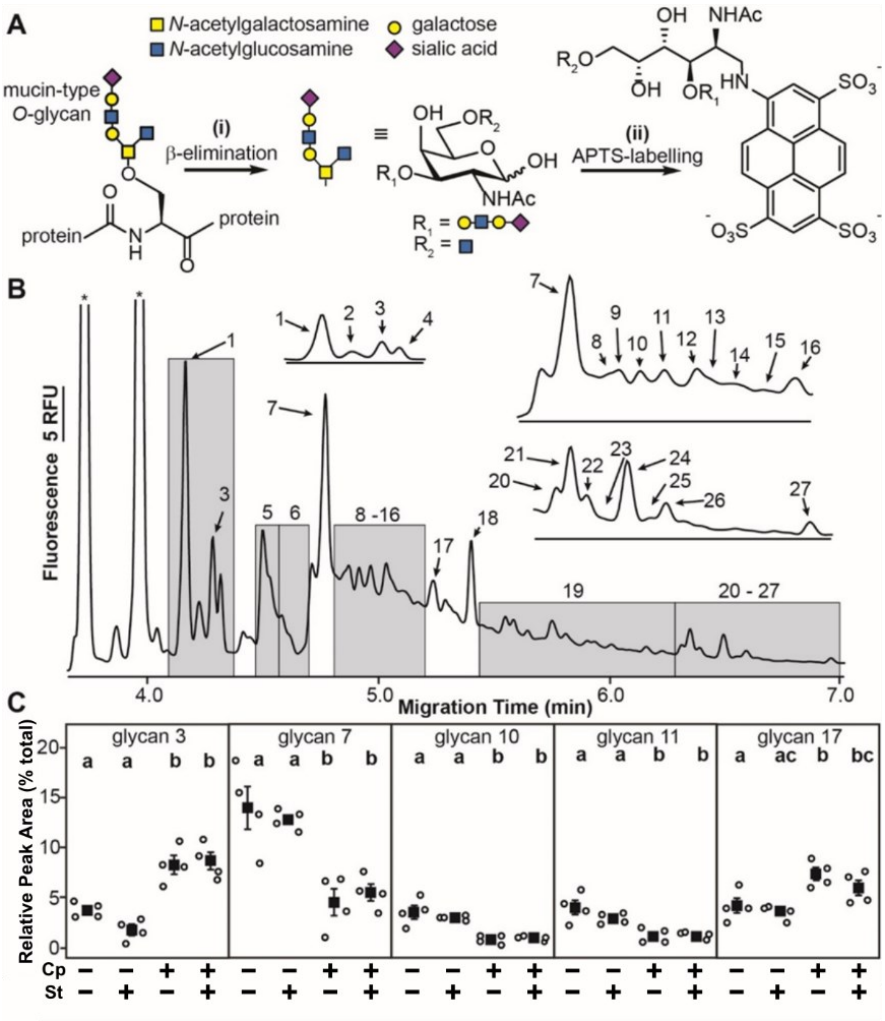

Figure 4.3 *Clostridium perfringens* induces alterations in mucus *O*-glycan profiles. Birds were administered 0.2% ethanol drinking water (Cp-St-), challenged with 10^7 CFU *C. perfringens* (Cp+St-), administered 20 mg/L CORT (Cp-St+), or received both *C. perfringens* and CORT challenge (Cp+St+). Treatments commenced in birds at 14-days-of-age, where *C. perfringens* was administered for 2 days and CORT for 7 days. (A) Freeze-dried mucus samples were β-eliminated under non-reducing conditions (i) to yield free, reducing *O*-glycans that could be fluorescently labelled with 8-aminopyrene-1,3,6-trisulfonate, and (ii) resolved by capillary electrophoresis (CE). (B) Resulting CE electropherograms revealed over 50 *O*-glycans per sample, 26 of which were manually integrated in order to calculate their abundances as a percent of the total detected *O*-glycans. Peaks labelled with asterisks are attributable to excess APTS reagent. (C) Strip chart of the *O*-glycans in which relative levels differ ($P < 0.050$) in one or more treatment group. Solid square markers denote group means, and the vertical lines associated with markers indicate standard error of the means. Markers not labelled with the same letter differ ($P \leq 0.050$).

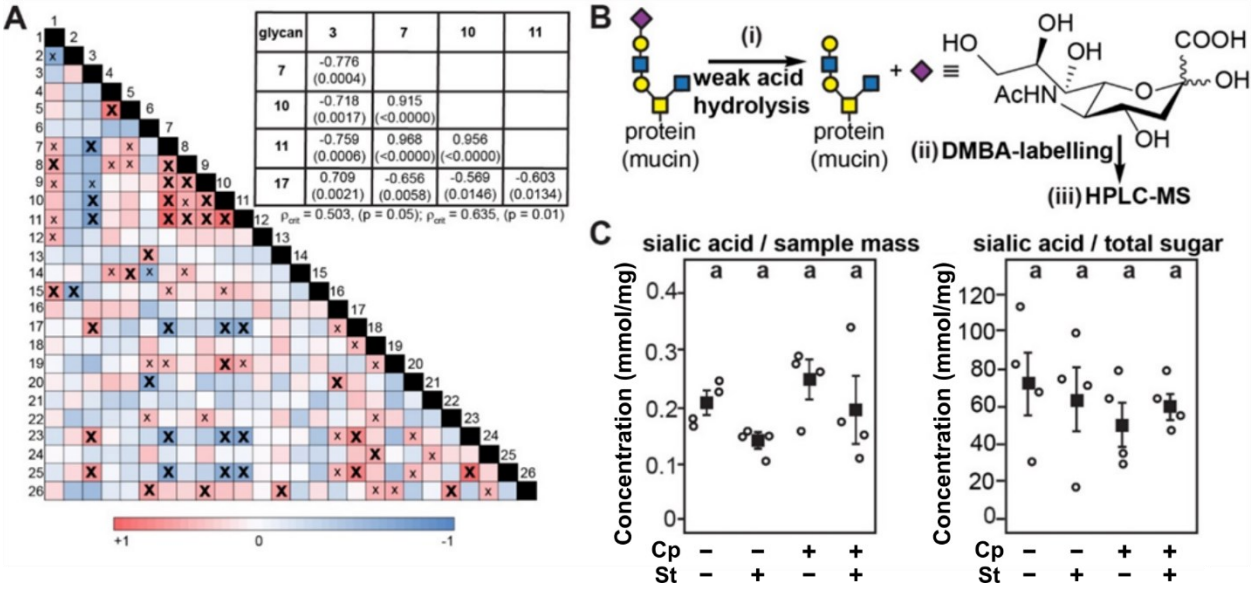

Figure 4.4 Sialic acid- and sulfate-containing *O*-glycans are inversely correlated but *Clostridium perfringens* infections and stress do not significantly affect total sialic acid levels. Birds were administered 0.2% ethanol drinking water (Cp-St-), challenged with 10^7 CFU *C. perfringens* (Cp+St-), administered 20 mg/L CORT (Cp-St+), or received both *C. perfringens* and CORT challenge (Cp+St+). Treatments commenced in birds at 14-days-of-age, where *C. perfringens* was administered for 2 days and CORT for 7 days. (A) A Spearman's rank correlation test was performed to determine significant associations between relative levels of all integrated *O*-glycans detected by CE (26) in all mucus samples (n = 16); x = ρ_{crit}<0.50 (P < 0.050) and X = ρ_{crit}<0.64 (P = 0.010). (B) Mucus samples were hydrolyzed (i), and labelled with 1,2-diamino-4,5-dimethylbenzene (DMBA; ii) to permit sialic acid quantitation by HPLC-MS (iii). (C) No significant differences in sialic acid levels were observed among the treatments, although stress tended to lower levels (as a fraction of mucus mass) in both Cp-St+ and Cp+St+ birds. Differing letter codes above each treatment indicate significant differences by the adjusted P-values produced by the HSD test.

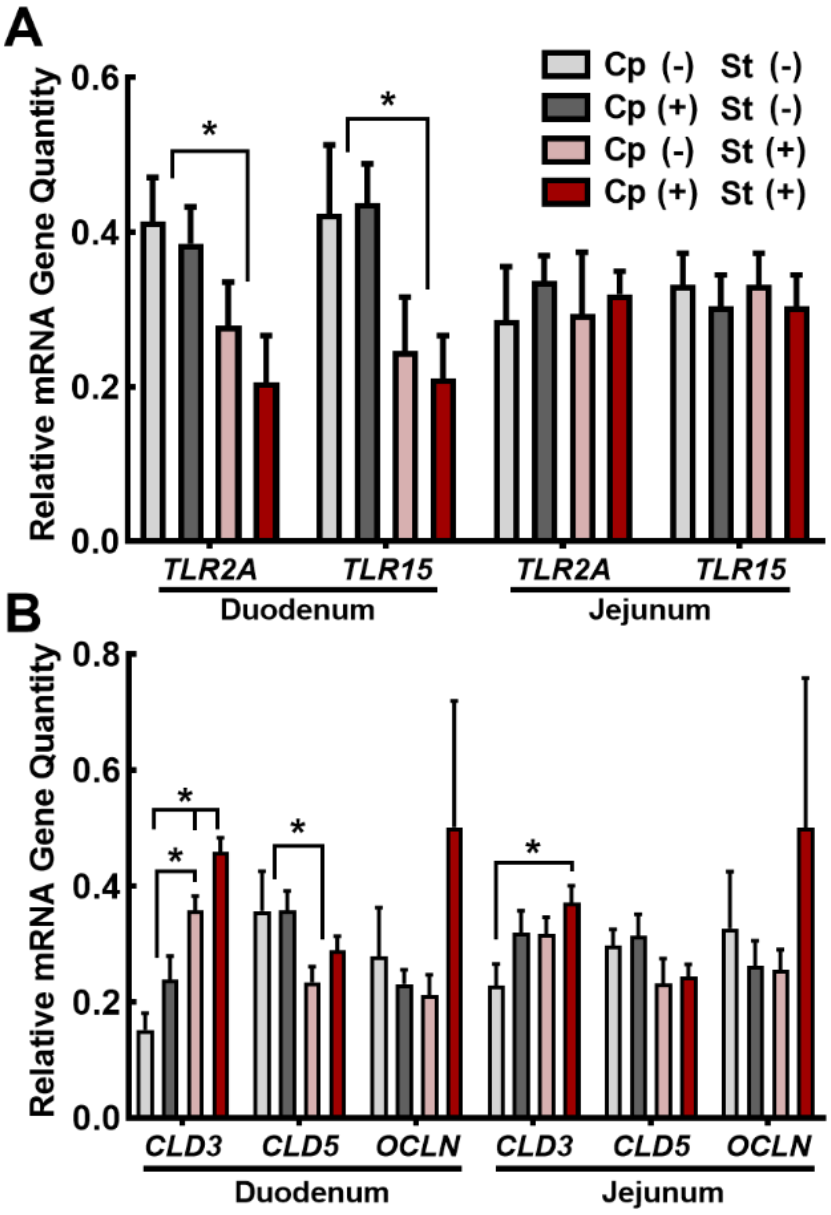

Figure 4.5 *Clostridium perfringens* challenge and corticosterone treatment modulated relative mRNA gene quantities associated with epithelial function in the small intestine. Birds were administered 0.2% ethanol drinking water (Cp-St-), challenged with 10^7 CFU *C. perfringens* (Cp+St-), administered 20 mg/L CORT (Cp-St+), or received both *C. perfringens* and CORT challenge (Cp+St+). Treatments commenced in birds at 14-days-of-age, where *C. perfringens* was administered for 2 days and CORT for 7 days. (A-B) Relative mRNA quantities in the duodenum and jejunum. (A) *TLR2A* and *TLR15* (B) *CLDN3*, *CLDN5*, and *OCLN*. Vertical lines associated with histogram bars represent standard error of the means (n = 4). Asterisks indicate significant differences ($P < 0.050$).

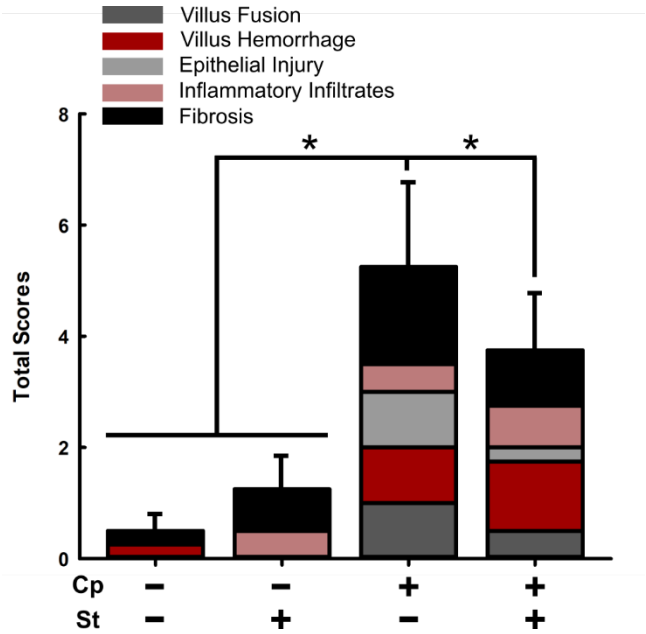

Figure 4.6 *Clostridium perfringens* incited histopathologic changes in the small intestine. Birds were administered 0.2% ethanol drinking water (Cp-St-), challenged with 10^7 CFU *C. perfringens* (Cp+St-), administered 20 mg/L CORT (Cp-St+), or received both *C. perfringens* and CORT challenge (Cp+St+). Treatments commenced in birds at 14-days-of-age, where *C. perfringens* was administered for 2 days and CORT for 7 days. Vertical lines associated with histogram bars represent standard error of the means for the total histopathologic score (n = 4). Asterisks indicate significant differences ($P < 0.050$).

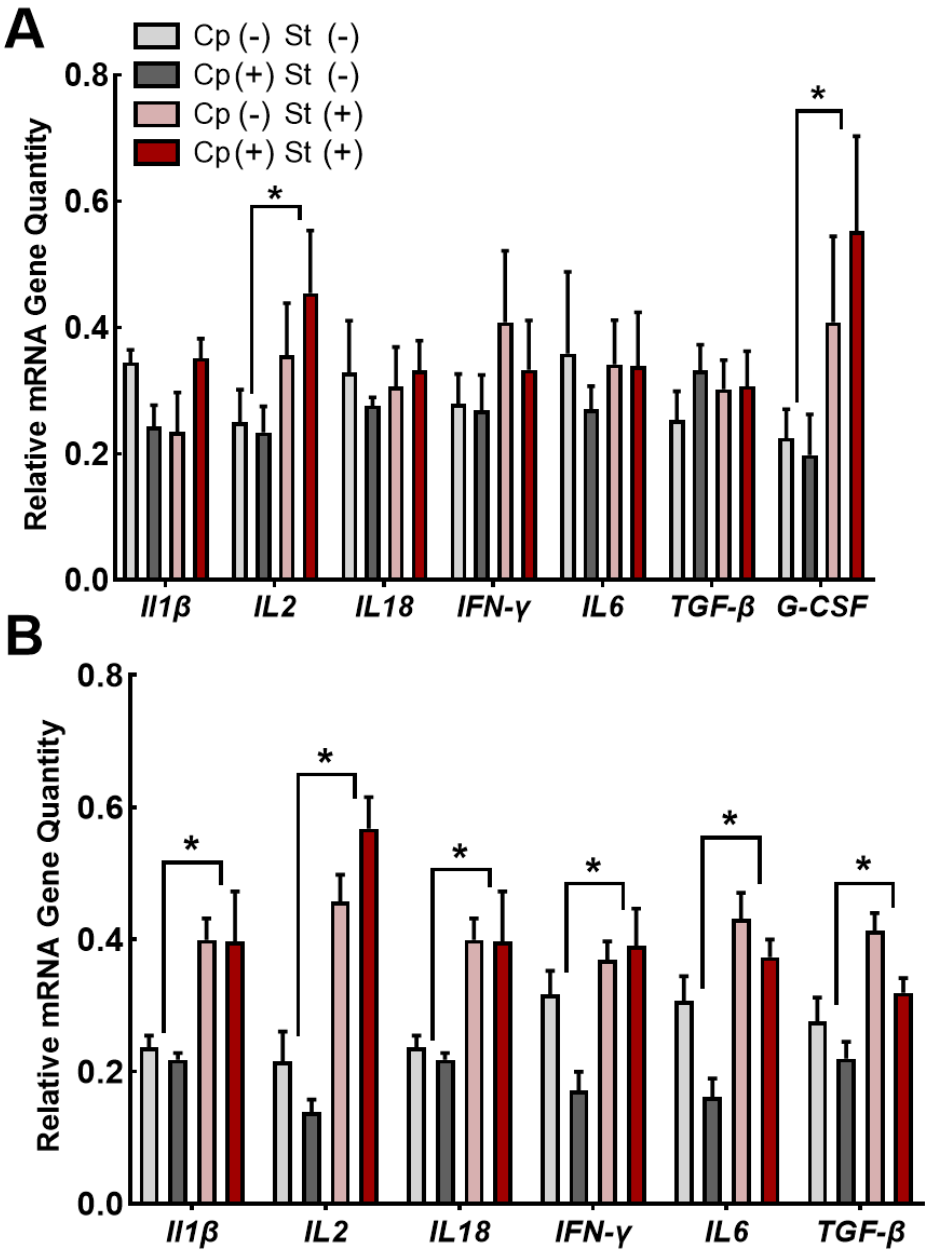

Figure 4.7 Corticosterone administration modulated relative mRNA gene quantities of immune cytokine genes in the spleen and thymus. Birds were administered 0.2% ethanol drinking water (Cp-St-), challenged with 10^7 CFU *C. perfringens* (Cp+St-), administered 20 mg/L CORT (Cp-St+), or received both *C. perfringens* and CORT challenge (Cp+St+). Treatments commenced in birds at 14-days-of-age, where *C. perfringens* was administered for 2 days and CORT for 7 days. (A-B) Relative mRNA quantities in the (A) spleen and (B) thymus. Vertical lines associated with histogram bars represent standard error of the means (n = 4). Asterisks indicate significant differences ($P < 0.050$).

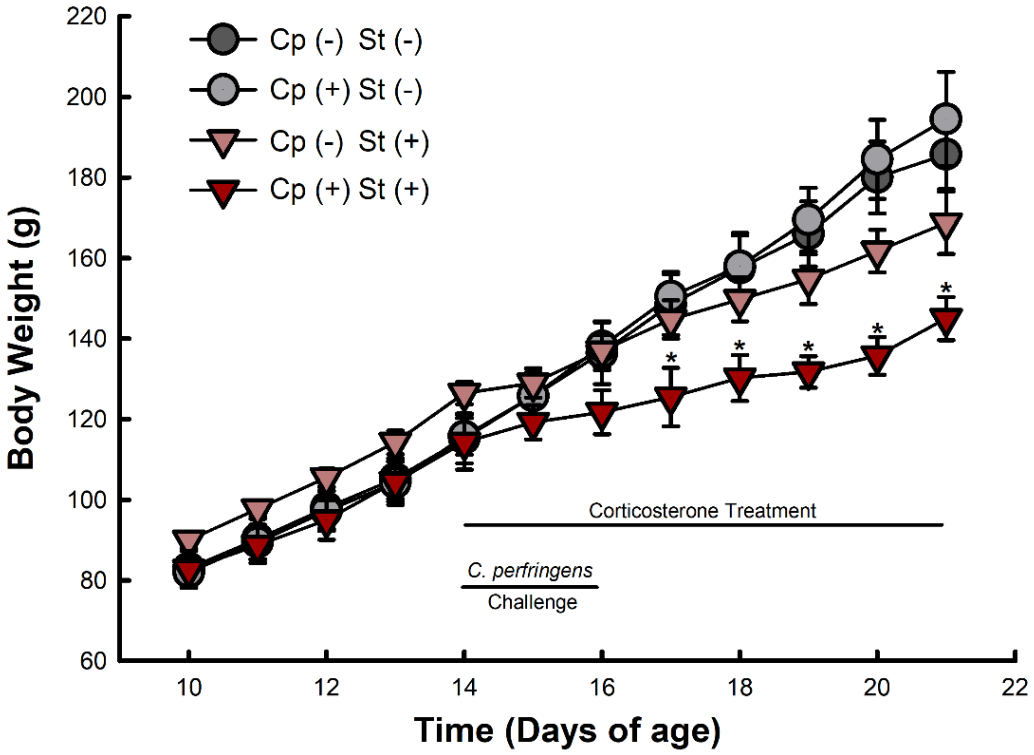

Figure 4.8 Birds co-challenged with *C. perfringens* and corticosterone exhibited decreased weight gain. Birds were weighed daily from 10 days post-hatch to the study endpoint. Birds were administered 0.2% ethanol drinking water (Cp-St-), challenged with 10^7 CFU *C. perfringens* (Cp+St-), administered 20 mg/L CORT (Cp-St+), or received both *C. perfringens* and CORT challenge (Cp+St+). Treatments commenced in birds at 14-days-of-age, where *C. perfringens* was administered for 2 days and CORT for 7 days. Vertical lines associated with markers represent standard error of the means (n = 4); markers without vertical lines indicates marker is obscuring the standard error mean or standard error of mean is too small to be represented. Asterisks indicate significant differences ($P < 0.050$) between the Cp+St+ treatment relative to Cp-St- and Cp+St- treatments.

Chapter 5: The development of a stress-induced model of necrotic enteritis in broiler chickens using acute dietary corticosterone administration

5.1 Abstract

Mounting evidence indicates that stress can predispose chickens to disease. The objective of the current study was to develop a method that utilized physiological stress to induce acute necrotic enteritis (NE) in Ross 308 broiler chickens. Stress was mediated through the administration of the stress hormone, corticosterone (CORT). At 11 days post-hatch (p.h.), CORT (20 mg/kg) administration commenced. At 12 and 13 days p.h., birds were orally inoculated with a virulent strain of *Clostridium perfringens*, and on 14 days p.h., birds were euthanized. Birds administered CORT exhibited decreased weight gain, and birds co-challenged with *C. perfringens* and CORT were affected to a higher degree. Necrotic lesions were present in birds inoculated with *C. perfringens* (33%), although a higher prevalence of birds treated with *C. perfringens* and CORT exhibited lesions (100%). *Clostridium perfringens* densities were correlated with necrotic lesions and histopathological scores. Both *C. perfringens* and CORT treatment altered mRNA responses. In this regard, birds infected with the pathogen showed higher relative mRNA concentrations of *TLR2A*, *TGFB2*, and *iNOS*. In birds administered CORT, *iNOS* and *TLR2A* mRNA concentrations were higher and lower, respectively. An interaction between *C. perfringens* challenge and CORT administration was observed for *TLR2A*. Findings suggest the involvement of CORT in suppressing immune stimulation in the epithelium, which may result in the manifestation of necrotic lesions in CORT treated birds. Overall, the CORT stress model resulted in levels of NE comparable to other models of NE that currently exist without the use of a co-infection agent. This model may facilitate the exploration of mechanisms of stress-induced NE.

5.2 Introduction

The increasing economic impacts of NE to poultry production has driven the need to develop models of necrotic enteritis (NE). Several predisposing states and conditions in the intestine have been described that contribute to the proliferation and success of infection by *C. perfringens.* Damaged mucosa, compromised barrier function, and mucus release can promote *C. perfringens* pathogenesis and are commonly induced through co-challenging birds with *Eimeria maxima* and *C. perfringens* [216]. Currently, the most common method of inducing NE in a laboratory setting is by utilizing *E. maxima* as a co-infection agent, in combination with a strain of *C. perfringens* that expresses cardinal virulence factors (e.g. NetB toxin) [114, 115]. Damage sustained to the epithelium by *E. maxima* can expose extracellular matrices, such as collagen, that *C. perfringens* can adhere to, and also facilitates invasion of epithelial cells [216, 217]. Moreover, *C. perfringens* possesses enzymes that breakdown mucin, and the

bacterium can grow in minimal media containing mucin glycoproteins [125, 205]. Diet, such as non-starch polysaccharides and protein rich diets, are also factors that can contribute to NE [114, 264]. Immunosuppression is another condition considered to be a predisposing factor to NE. In this regard, infectious bursal disease virus vaccine has been used as a method to promote immunosuppression and induce NE [265]. All of these predisposing conditions in the intestine undoubtedly alter the microbiota [216]. Modulations to the microbiota have been described with NE, although factors that contribute to its alteration are complex [191]. Research models of NE often fail to reproduce disease as severe as occurs in field cases, which may be due to the multitude of predisposing conditions to NE that occur on farm. This highlights the need for research to continue identifying factors that predispose birds to NE and to elucidate mechanisms of disease onset.

Increasingly research is beginning to verify physiological stress as a predisposing factor to NE. Stress can promote similar predisposing conditions in the intestine as other co-factors to disease. For example, stress has been implicated to impact barrier function, and may alter mucus secretion [93, 221, 224]. It is well recognized that stress, especially chronic stress, can result in the suppression of immune responses and promote the development of disease [4]. Heat stress has shown variable results on NE [215, 266]. For instance, cyclic acute heat stress tended to promote disease, while chronic heat stress reduces macroscopic lesions [215, 266]. Moreover, various stressors encountered in production settings, such as stocking density and cold temperatures, have shown to increase macroscopic necrotic lesions, although the mechanisms' response are yet to be determined [130, 131].

I previously administered corticosterone (CORT) in water to layer chickens as a method to mediate a physiological stress response and characterize the impacts of subclinical NE on the host and the enteric microbiota [193]. In the current study, I hypothesize that broiler chickens administered CORT will mount a stress response that will predispose them to clinical NE due to a reduction in immune responses. To test this hypothesis, the objective of the current study was to examine microscopic, macroscopic, and molecular changes in the intestines of broiler chickens ± administration of dietary CORT and ± *C. perfringens* challenge. A primary goal of the study was to develop a stress-induced model of NE for future elucidation of mechanisms of stress-incited predisposition of birds to NE, and to develop rationale-based mitigation strategies including alternatives to antibiotics.

5.3 Materials and Methods

5.3.1 Ethics statement

The study was carried out in strict accordance with the Canadian Council on Animal Care Guidelines. The project was reviewed and approved by the Lethbridge Research and Development

Centre (LeRDC) Animal Care Committee (Animal Use Protocol Review # 1912) before commencement of the research.

5.3.2 Experimental design

The experiment was designed as a factorial experiment with two levels of CORT administration (±) and two levels of *C. perfringens* challenge (±) arranged as a complete randomized design with six replicates. The four treatments were: (1) no CORT and no *C. perfringens* treatment (Control); (2) no CORT treatment and positive *C. perfringens* challenge (*Cp*); (3) positive CORT treatment and no *C. perfringens* challenge (CORT); and (4) *C. perfringens* treatment and positive CORT (*Cp* + CORT). The experiment was repeated on three separate occasions with two replicates per run.

5.3.3 Animals and husbandry

Ross 308 broiler chicken eggs were obtained from a local hatchery (Lethbridge, AB, CA). Eggs were incubated in a Brinsea Ovation 56 EX fully automatic digital egg incubator (Brinsea Products Inc., Titusville, FL) according to the manufacturer's guidelines for incubating chicken eggs. Eggs were maintained at 37.5°C at 60% humidity. Eggs were turned hourly for the first 18 days of incubation. Thereafter, eggs were set flat for hatching, and humidity was increased to 70%. Chicks (1-day-old) were placed in pairs within individually ventilated cages (1,862 cm^2 floor space; Techniplast, Montreal, QC). These cages were operated in containment mode (i.e. negative air pressure flow) to provide bi-directional HEPA filtered air exchange and protect researchers from pathogens, including *C. perfringens*. Birds were provided continuous access to a starter diet for the first 10 days and a grower diet from day 11 until the end of the experiment (Table 5.1). Birds had access to water at all times through two nipple drinkers per cage. Birds were maintained at 30°C for 2 days, 28°C for 2 days, then maintained at 26°C for the remainder of the experiment on a 18 hr light: 6 hr dark cycle. Birds were weighed daily.

5.3.4 Corticosterone administration and diet

Corticosterone was incorporated into the grower diet at a dose of 20 mg/kg. Fresh feed was given to birds each morning and afternoon. Corticosterone administration began on day 11 post-hatch (p.h.) and continued until the end of the experiment.

5.3.5 *Clostridium perfringens* inoculation

A starter culture of *C. perfringens* (strain CP1) was grown overnight at 37°C in Heart Infusion Broth and incubated within an anaerobic chamber containing an 85% N_2 : 10% CO_2 : 5% H_2 atmosphere. The following morning, 2.5 mL of the overnight culture was transferred to 50 mL of Fluid Thioglycolate Medium within the anaerobic chamber, and bacterial cells were incubated for 4 hr at 37°C. Birds were inoculated with 1 mL of *C. perfringens* containing 1-2 x 10^8 CFU via oral gavage starting at 12 days p.h.

for two consecutive days. Cell density of *C. perfringens* was enumerated by diluting inoculation broth in a 10-fold dilution series with 200 µL spread onto Columbia agar containing 5% sheep blood. Cultures were maintained in an anoxic atmosphere at 37°C for 24 hr, and colonies were counted at the dilution yielding 30 to 300 CFU.

5.3.6 Animal euthanasia and tissue collection

All birds were anesthetized, euthanized, and sampled at 14 days p.h. Birds were anesthetized with isoflurane (5% isoflurane; 1 L O_2/min) and euthanized by cervical dislocation while under general anesthesia. The abdomen was opened, and the small intestine was removed aseptically for examination and lesion scoring. Digesta from the jejunum was removed using a sterile wooden splint and stored at -80°C until processed. Jejunal samples collected for RNA analysis were placed in RNAlater™ Stabilization Solution (Thermo Scientific, Ottawa, ON) and stored at -80°C until processed. Tissues for histopathology were placed in 10% neutral buffered formalin (Leica, Concord, ON).

5.3.7 Lesion scoring and histopathology

The entire length of the small intestine (duodenum to ileal-cecal junction) was examined for gross lesions and scored 0-6 as described in Shojadoost et al. 2012 [114]. The proximal jejunum region in all birds was obtain for histopathogical examination; care was taken to ensure that lesions were not sampled. Proximal jejunum tissue was harvested from a measured location of approximately 5 cm from the terminal region of the distal duodenal loop to ensure consistency between tissue sampling. Jejunal tissue was fixed for a minimum 24 hr before being dehydrated, embedded in paraffin, and sectioned. Samples were dehydrated using a Leica tissue processor (Leica TP1020 Benchtop Tissue Processor, Leica Biosystems, Concord, ON), embedded in paraffin blocks using a Shandon Histocentre 3 Embedding Center (Thermo Scientific), and sectioned (≈5 µm) using a Finesse 325 Manual Rotary Microtome (Thermo Scientific). Slides were deparaffinized with xylene and stained with hematoxylin and eosin. Sections were scored by a pathologist (V.F.B) blinded to treatments using a modified scoring system based on previously described methods [121, 234, 267]. Sections were graded from 0 to 4 for villus fusion, villus atrophy, mucosal necrosis, bacterial invasion, and lamina propria changes (Table 5.2). Total histological scores were determined by calculating the sum of scores from all categories.

5.3.8 Quantitative PCR for *Clostridium perfringens*

DNA was extracted from jejunal digesta using a Qiagen DNA Fast Stool Kit (Qiagen Inc.). To each sample, 200 mg of 0.5-mm-diameter glass beads were added at the lysis step. The sample with beads was vortexed for 3 min, and then homogenized using Qiagen TissueLyser I (Qiagen Inc.) for 3 min at 50 Hz. A standard curve of known copies of 16S DNA specific to *C. perfringens* was generated as previously

described [101, 193]; the standard curve was generated using a 10-fold dilution series ranging from 10^7 to 10^1 copies of the *C. perfringens* 16S rRNA gene. Each reaction contained 10 µL Quantitect SYBR green master mix (Qiagen Inc.), 1 µL of each primer, 2 µL of bovine serum albumin, 4 µL of DNase-free water, and 2 µL of template DNA (10 ng/µL). Reaction conditions were: 95°C for 15 min; and 40 cycles of 95°C for 15 s, 55°C for 30 s, and 72°C for 30 s. Melt curve analysis was conducted from 55-95°C. A MxPro 3005P thermocycler (Agilent Technologies, Santa Clara, CA) was used to conduct qPCR analysis. Each reaction was run in duplicate, and the mean of the two observations was calculated.

5.3.9 Quantification of jejunal mRNA

RNA was extracted from jejunal tissue using RNeasy Plus Mini Kit (Qiagen Inc., Toronto, ON). An Agilent Bioanalyzer (Agilent Technologies, Mississauga, ON) was used access the quality and quantity of RNA, and 1 µg of RNA was reverse transcribed to cDNA using a QuantiTect reverse transcription kit (Qiagen Inc.). A MxPro 3005P thermocycler (Agilent Technologies) was used to perform quantitative PCR (qPCR). Each reaction contained 5 µL of Quantitect SYBR green master mix (Qiagen Inc.), 0.5 µL of each primer (10 µM), 3 µL of RNase-free water, and 1 µL of cDNA. Reaction conditions were: 95°C for 15 min; 40 cycles of 95°C for 15 sec, 55-58°C for 30 sec, 72°C for 30 sec. Melt curve analysis was conducted from 55 to 95°C. Primer sequences and annealing temperature are listed in Table 5.3; primers were designed using NCBI primer BLAST unless cited otherwise. Reactions were run in duplicate and the average ct values were used to calculate mRNA concentrations relative to the reference gene, β-actin, using qBase+ software (Biogazelle, Gent, Belgium) [158].

5.3.10 Statistical analyses

Graphpad Prism software (La Jolla, CA, USA) was used to perform statistical analysis. Continuous data (i.e. weight gain, mRNA gene expression, and *C. perfringens* densities) was checked for normality and analyzed by two-way analysis of variance (ANOVA) with Tukey multiple comparison test. Non-parametric data (i.e. necrotic lesion and histopathological scoring) was analyzed using the Kruskal-Wallis test with Dunn's test for multiple comparison correction. Correlation analysis to examine the relationship between necrotic lesion and histopathological scoring was conducted using Spearman's correlation. The relationship between *C. perfringens* densities and necrotic lesion/histopathological scores were assessed using point biserial correlation [268].

5.4 Results

5.4.1 Corticosterone administration reduced bird weight gain

No difference in weight gain was observed among treatments from days 1 to 10 (Table 5.4). Birds administered CORT ($P \leq 0.014$) and *Cp* + CORT ($P \leq 0.003$) exhibited a reduction in weight gain in

comparison to other treatments from days 11 to 14 once CORT and *C. perfringens* treatments commenced.

5.4.2 *Clostridium perfringens* challenge induced gross and histopathological changes in the jejunum

Necrotic lesions (Figure 5.1A) were observed only in *Cp* (33.3%) and *Cp* + CORT (100%) treatment birds (Figure 5.1B). *Cp* challenge increased lesions scores ($P < 0.001$). Pairwise comparisons showed that only *Cp* + CORT birds had higher lesions scores ($P = 0.002$) in comparison to the Control and CORT treatment birds (Figure 5.1C). Villus fusion ($P < 0.010$), villus atrophy ($P < 0.010$), mucosal necrosis ($P < 0.024$), bacterial invasion ($P < 0.028$), and total histopathological scores differed ($P \leq 0.033$) in *C. perfringens* challenged birds (*Cp* and *Cp* + CORT treatment) (Figure 5.1D). There was a positive correlation ($P = 0.010$, $r=0.514$) between necrotic lesion and histopathologic scoring.

5.4.3 *Clostridium perfringens* densities correlated with necrotic lesion and total histopathological scores

The jejunum of birds inoculated with *C. perfringens* were colonized by the pathogen (Figure 5.2A). Birds challenged with *Cp* ($P \leq 0.023$) and *Cp* + CORT ($P \leq 0.001$) showed higher densities of *C. perfringens* in jejunum digesta in comparison to Control and CORT birds. Point biserial correlation analysis showed that *C. perfringens* densities were correlated with necrotic lesion scores ($r = 0.900$, two-tailed $P < 0.001$; Figure 5.3B) and total histopathological scores ($r = 0.737$, two-tailed $P < 0.001$; Figure 5.3C).

5.4.4 Corticosterone and *Clostridium perfringens* modulated immune responses in the jejunum

No difference ($P \geq 0.830$) in mRNA quantity of *IL2* or *IL17* among treatments was observed (Figure 5.3A-B). *Clostridium perfringens* treatment tended to increase relative mRNA quantities of *IL1B*, although not consistently (Figure 5.3C; $P = 0.100$). Quantities of *TGFB2* mRNA were higher ($P = 0.025$) in birds inoculated with *C. perfringens* (Figure 5.3D). Both the *C. perfringens* ($P = 0.041$) and CORT ($P = 0.050$) treatments altered *iNOS*, but they did so independent of each other ($P = 0.250$; Figure 5.3E). An interaction ($P = 0.050$) between *C. perfringens* and CORT was observed for *TLR2A* (Figure 5.3F). This was due to *Clostridium perfringens* challenge stimulated *TLR2A* ($P = 0.013$), whereas, CORT treatment decreased *TLR2A* ($P = 0.031$).

5.5 Discussion

The overarching goal of the current study was to examine the impact of controlled physiological stress exposure (i.e. as a predisposing factor) to achieve acute NE manifestation in broiler chickens for subsequent use as a high prevalence model of disease. Birds were administered CORT in feed to mediate a stress response, and then challenged with a virulent strain of *C. perfringens* (CP1). Birds were examined for necrotic lesion scoring and histopathological changes in the small intestine to ascertain the

degree of disease. All *C. perfringens* challenged birds showed necrotic lesions and higher histopathological scores, although birds co-challenged with *C. perfringens* and CORT exhibited a higher prevalence of necrotic lesions than birds not administered CORT. Densities of *C. perfringens* in the jejunum were correlated with both necrotic lesion and histopathological scores. Moreover, both *C. perfringens* and CORT treatments modulated mRNA gene expression in the jejunum.

5.5.1 Corticosterone treatment impacts weight gain

NE imparts economic losses through mortality, but also through poor bird performance in subclinical forms of the disease [113]. My previous research in layer birds showed that subclinical NE was associated with reduced bird weight, but only in birds inoculated with *C. perfringens* CP1 and subjected to corticosterone-induced stress [193]. In the current study, CORT administration was found to impart the greatest impact on bird weight gain, as birds co-challenged with *C. perfringens* and CORT showed the largest reduction in weight gain. This is consistent with other studies that have demonstrated that *C. perfringens* challenge alone does not affect weight gain, and that other variables in combination with the pathogen are necessary to impact performance [193, 257]. In this regard, co-infection with *E. maxima*, which can promote immunosuppression and intestinal damage, was necessary to incite infection and result in weight gain losses [216, 265]. These impacts to bird health can contribute to increased disease severity and reduced weight gain [113].

5.5.2 Corticosterone treatment induced a higher prevalence of necrotic enteritis

Studies developing models of NE rely on the onset and severity of necrotic lesions as a cardinal metric to evaluate efficacy. An advantage of lesion scoring, in comparison to histopathological changes, is the ability to survey the entire small intestine for disease. Necrotic lesions were observed only in a subset of birds inoculated with *C. perfringens*, whereas all the birds challenged with CORT and *C. perfringens* showed at least one necrotic lesion. The finding that physiological stress induction exacerbates disease is supported by other research that showed that stocking density and cold stress increased the prevalence of necrotic lesions following *C. perfringens* challenge [130, 131]. Treatment with CORT and *C. perfringens* resulted in consistent lesion development in all of the birds examined in the current study which indicates that this stress predisposition model could be useful to elucidate mechanisms (e.g. without the confounding impacts of a co-infection) and evaluate mitigation strategies.

Although the majority of published studies use lesion scoring as the sole metric of disease onset, some studies have evaluated the small intestine for histopathologic changes, and this can provide crucial information beyond evaluation of macroscopic lesions. For example, birds challenged with *C. perfringens* alone but not CORT showed higher histological scores in the jejunum relative to control animals, despite

showing limited development of necrotic lesions. This can indicate inflammation on a microscopic level indicative of subclinical NE. Furthermore, it demonstrates the stimulation of immune responses necessary to overcome or limit infection. In the present study, higher histopathological scores corresponded with elevated immune responses (i.e. *TLR2A*, *iNOS*, *TGFB2*) in *C. perfringens* challenged birds. From a performance perspective, mounting an immune response (i.e. in the absence of clinical disease) is still catabolically costly, which can adversely affect production. Histopathologic evaluation also allows researchers/clinicians to ascertain why acute disease was not manifested (i.e. to rule out lack of infection, virulence, or the colonization by *C. perfringens*).

5.5.3 *Clostridium perfringens* and corticosterone treatment altered immune responses in the jejunum

Six genes involved in immune function were measured in the jejunum. No changes were observed in the expression of the inflammatory genes, *IL2*, *IL17*, and *IL1B*, although a trend for higher *IL1B* mRNA quantities were observed in birds challenged with *C. perfringens*. A relative increase of *TGFB2* mRNA was observed in jejunal tissue following *C. perfringens* challenge. This is consistent with the observation of microscopic and macroscopic damage to the epithelium in *C. perfringens* challenged birds, as *TGFB* functions to promote tissue repair after injury [186]. *Clostridium perfringens* challenge increased *iNOS* mRNA expression, which is justified as *iNOS* secretion is stimulated by immune cytokines and bacterial infection [269]. Additionally, co-infection of *C. perfringens* and *E. maxima* has been shown to increase *iNOS* expression [270]. It was observed that the administration of CORT alone also increased relative quantities of *iNOS* mRNA. This is consistent with other research that showed that cold stress could incite increases in *iNOS* mRNA in the duodenum of broilers when cold stress persisted for 5 to 20 days [271]. In the current study, *TLR2A* mRNA increased with *C. perfringens* challenge, and this was predicted as *TLR2* has previously been shown to be stimulated in the intestinal epithelium of birds following infection by *C. perfringens* [250, 272]. Conversely, birds treated with CORT exhibited decreased quantities of *TLR2A* mRNA. This corresponds with my previous work in layer chickens which demonstrated CORT administration could decreased the expression of *TLR2A* and *TLR15* in the small intestine [193]. The downregulation of *TLR2* could be an indication of how gross pathologies were more prevalent in birds co-challenged with *C. perfringens* and CORT. For example, *TLR2* is a mediator of nuclear factor κB signaling, which leads to the activation of inflammatory responses [250]. Therefore, inadequate stimulation of *TLR2* in birds administered CORT could result in failed recruitment of immune cells and responses to the site of infection. This would permit *C. perfringens* to grow, colonize, and incite damage to the mucosa, leading to gross pathologies (i.e. necrotic lesions). Additionally, it has previously been demonstrated that *TLR2* activation can promote cell survival and inhibit apoptosis in *ex vivo* murine

intestinal epithelial cells [250]. Thus, an inactivation of *TLR2* may promote the opposite trend and facilitate the development of necrosis. Given the evidence of stress-incited predisposition of broiler chickens to NE that was obtained in the current study, further examination of how stress impacts immune response and other factors, such as mucus and tight junction formation, warrants additional investigation.

5.5.4 Conclusion

A high prevalence of clinical NE was achieved in broiler chickens inoculated with *C. perfringens* and administered dietary CORT to incite a defined physiological stress response. Birds receiving *C. perfringens* and CORT showed more consistent and severe development of necrotic lesions in comparison to birds challenged only with *C. perfringens*. Densities of *C. perfringens* correlated to necrotic lesion and histopathologic scores in the jejunum. Increased relative concentrations of *TGFB2* and *iNOS* mRNA following *C. perfringens* challenge indicated that infection occurred. The reduction in quantified *TLR2A* mRNA in birds co-challenged with C. *perfringens* and CORT suggested stress-incited impairment of immune intervention in the small intestinal epithelium, which may be a key mechanism by which stress promotes gross pathologies. This warrants further investigation. Importantly, the CORT model generated consistent clinical NE in broilers, and at a comparable level to other NE models. Although the CORT model may not be suitable for researchers testing NE vaccines, it does not require a co-infection agent (i.e. *Eimeria* spp.). Thus, the model may facilitate the elucidation of mechanisms, including on how stress predisposes birds to NE. Moreover, the model may be useful to discovery of biomarkers (e.g. of disease predisposition), and to advance antimicrobial alternatives.

5.6 Tables and Figures

Table 5.1 Starter and grower diet ingredients and rations.

	Starter (0 to 10 d-of-age)	**Grower** (11 to 14 d-of-age)
Ingredient	**% of mix**	**% of mix**
Corn	49.53	54.68
Soybean meal	43.06	37.31
Canola oil	2.39	3.26
Salt	0.51	0.52
Limestone	1.52	1.41
Dicalcium phosphate	1.26	1.09
Magnesium oxide	0.1	0.15
L-Lysine HCl	0.11	0.12
D,L-Methionine	0.37	0.33
L-Threonine	0.15	0.13
Vitamin premix*	0.5	0.5
Choline premix	0.5	0.5

*Corticosterone was mixed in the vitamin premix in grower diet to attain 20 mg/kg dose in diet

Table 5.2 Histopathologic scoring criteria*.

Parameter
Villus Fusion
1-occasional fusion of two villi in a section
2-occasional fusion > 2 villi or several fusions of two villi
3-multiple areas where > 2 villi were fused
4-large clusters of fused villi throughout
Villus Atrophy
1-slight
2-slight to moderate
3-moderate to severe
4-severe
Mucosal Necrosis
1-necrosis or sloughing of the mucosal epithelium
2-scattering of necrotic foci
3-multiple necrotic foci
4-coalessced or layered necrosis
Bacterial Invasion
1-small number of bacteria
2-sporadic clumps of bacteria
3-multiple clumps of bacteria
4-large clumps of bacteria
Lamina Propria Changes
1-mild edema and a few cells showing early necrosis
2-amorphous eosinophillic material replacing area in lamina propria
3-obliteration of structural integrity of lamina propria
4-coagulative necrosis with band of neutrophils

*scoring criteria collated by Dr. Valerie Boras based on [121, 234, 267]

Table 5.3 List of primers used to quantify *Clostridium perfringens* and relative mRNA concentrations.

Name	Abbreviation		Sequence 5' to 3'	Product Size	Reference Sequence	Source
C. perfringens (16S)	*CP1.2*	F	AAAGATGGCATCATCATTCAAC	283		PMID: 12399288 **[199]**
		R	TACCGTCATTATCTTCCCCAAA			
Interleukin 2	*IL2*	F	TAACTGGGACACTGCCATGA	93	NM_204153.1	PMID: 32391086 [193]
		R	GATAGAGATGCTCCATAAGCTGT			
Interleukin 1β	*IL1B*	F	TGCCTGCAGAAGAAGCCTCG	137	NM_204524.1	PMID: 32391086 [193]
		R	CTCCGCAGCAGTTTGGTCAT			
Interleukin 17	*IL17*	F	AGATGCTGGATGCCTAACCC	154	NM_204460.1	This Study
		R	GTGGTCCTCATCGATCCTGTAA			
Transforming Growth Factor β2	*TGF*β2	F	CCATCTACAACAGCACCAGGG	157	NM_001031045.3	PMID: 32391086 [193]
		R	TAGCTTGGTGGGATGGCATTTTC			
Inducible Nitric Oxide Synthase	*iNOS*	F	GAACAGCCAGCTCATCCGATA	103		PMID: 17709416 [273]
		R	CCCAAGCTCAATGCACAACTT			
Toll-like receptor 2A	*TLR2A*	F	CAGCACAAGAGGCGTTCA	100	NM_204278.1	PMID: 32391086 [193]
		R	AACATTTTGGTGTAGCTGAGATG			
β-Actin	*BA*	F	CTCTGACTGACCGCGTTACT	172	NM_205518.1	PMID: 31848364 [36]
		R	TACCAACCATCACACCCTGAT			

Table 5.4 Average bird weight gain.

Treatment	Average Weight Gain (g ± SEM)		
	Day 1-5	**Day 6-10**	**Day 11-14**
Control	91.3 ± 6.4	178.5 ± 6.2	156.2 ± 5.2
Cp	95.7 ± 4.9	183.0 ± 4.5	154.3 ± 5.2
CORT	94.6 ± 9.0	167.7 ± 14.4	112.0 ± 11.8*
Cp + CORT	92.0 ± 8.0	179.5 ± 9.6	103.0 ± 11.0**

* $P \leq 0.014$ and ** $P \leq 0.003$ in comparison to the Control and *Cp* treatments

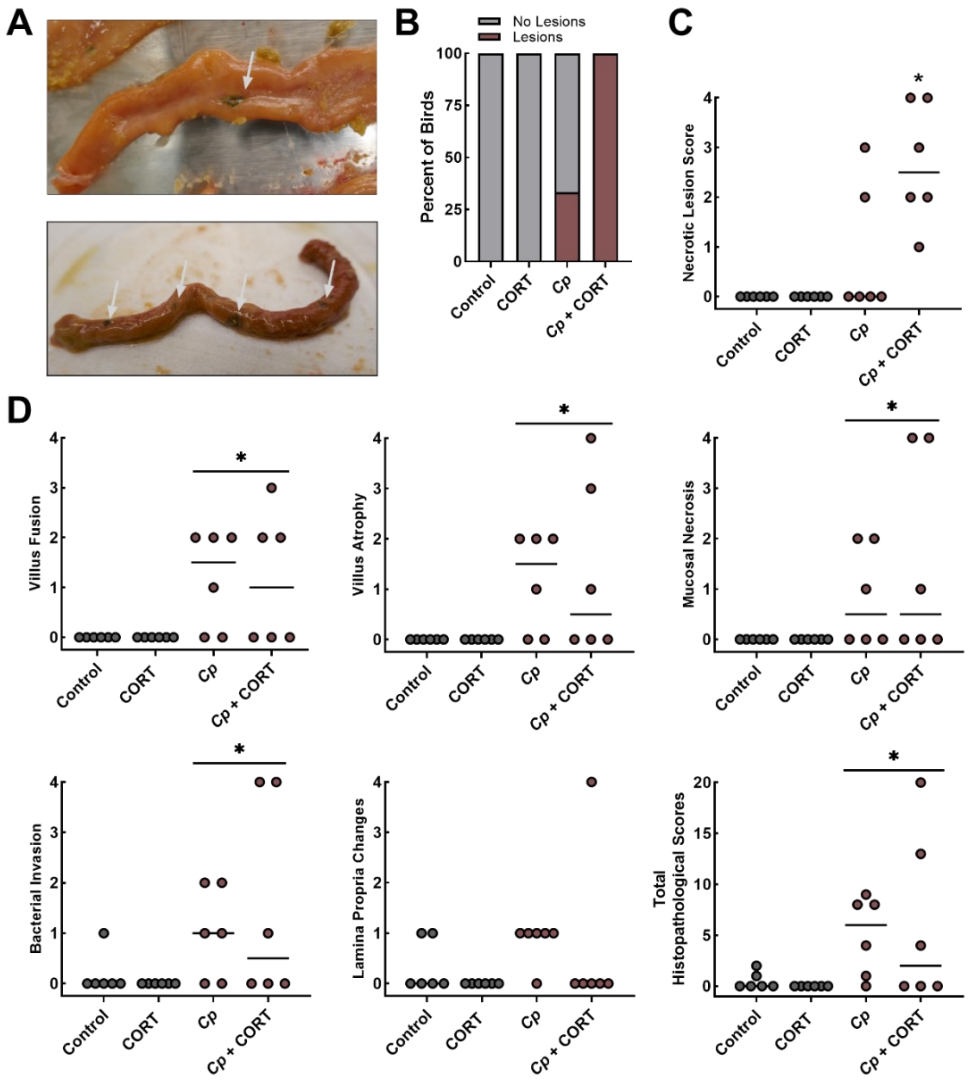

Figure 5.1 *Clostridium perfringens* incited gross and histopathological changes in the jejunum. Birds were untreated (Control), administered 20 mg/kg CORT in feed (CORT), challenged with *C. perfringens* (*Cp*), or received both CORT and *C. perfringens* treatment (*Cp* + CORT). CORT treatment commenced at 11 days post-hatch (p.h.). Birds were orally administered 1-2 x 10^8 CFU *C. perfringens* on days 12 and 13 p.h., and necropsies were conducted on day 14 p.h. (A) Pictures showing representative necrotic lesions in the proximal jejunum from *Cp* + CORT treated birds. (B) Histogram illustrating the prevalence necrotic lesions (≥ one lesion). (C) Gross necrotic lesion scores. Horizontal lines are the median (n = 6). Lesion scoring was executed as described Shojadoost et al. [114]. (D) Histopathological scores for villus fusion, villus atrophy, mucosal necrosis, bacterial invasion, and lamina propria changes. Total scores were calculated by taking the sum of all scores from each category. Horizontal lines are the median score (n = 6). Asterisk indicated $P < 0.050$ in comparison to the Control and CORT treatments.

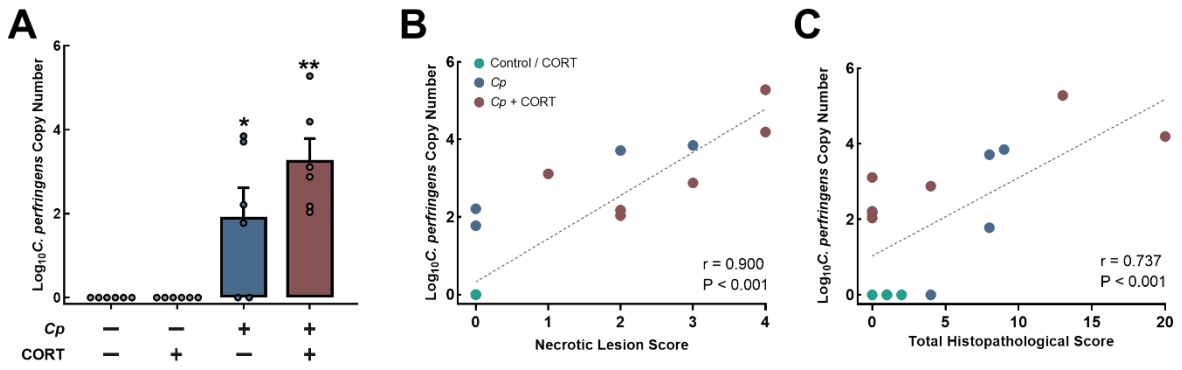

Figure 5.2 *Clostridium perfringens* densities in the jejunum. Birds were untreated (Control), administered 20 mg/kg CORT in feed (CORT), challenged with *C. perfringens* (*Cp*), or received both CORT and *C. perfringens* treatment (*Cp* + CORT). CORT treatment commenced at 11 days post-hatch (p.h.). Birds were orally administered 1-2 x 10^8 CFU of *C. perfringens* on days 12 and 13 p.h., and necropsies were conducted on day 14 p.h. (A) Densities of *C. perfringens* were determined by qPCR in jejunal digesta using primers specific for the 16S rRNA gene of *C. perfringens*. (B-C) Point biserial correlation of (B) necrotic lesion scoring and *C. perfringens* densities, and (C) total histopathological scores and *C. perfringens* densities. Vertical lines associated with histogram bars represent standard error of the means (n = 6). * $P \leq 0.023$ and ** $P < 0.001$ in comparison to control and CORT treatments.

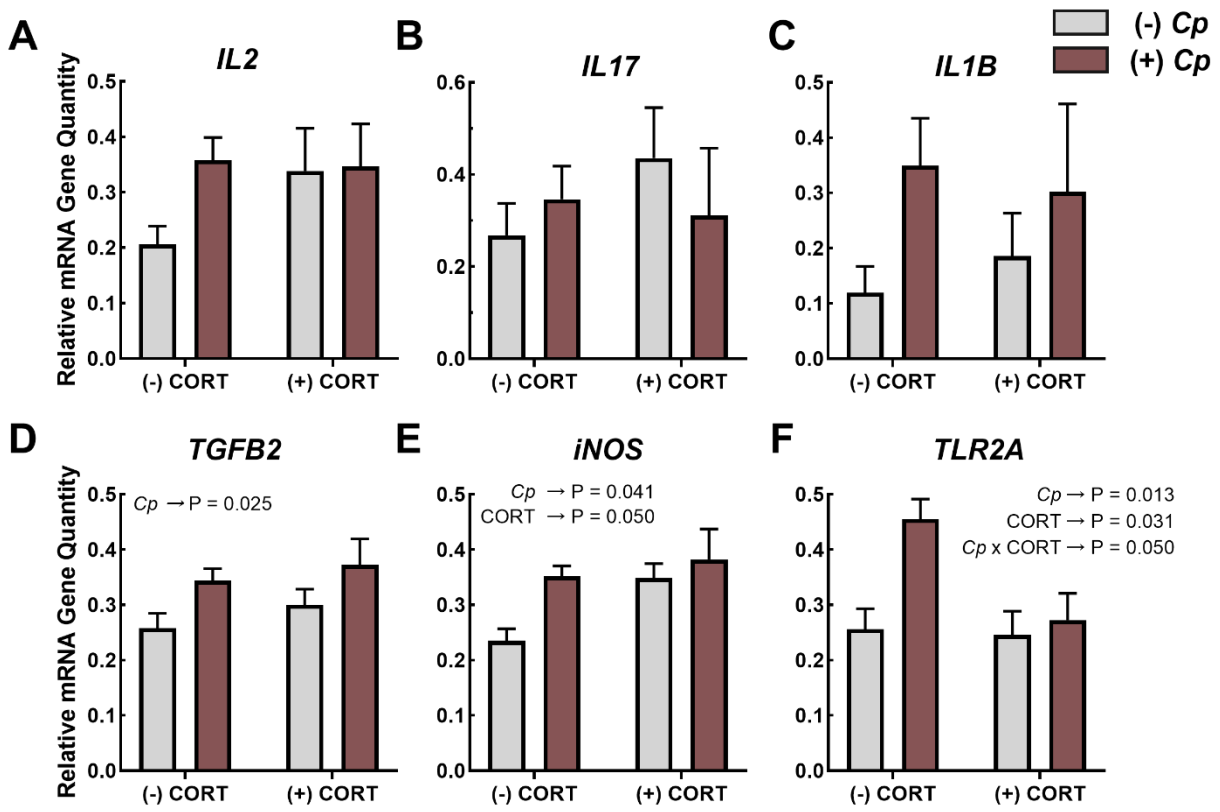

Figure 5.3 *Clostridium perfringens* and CORT modulate relative mRNA gene quantities in the jejunum. Birds were untreated (Control), administered 20 mg/kg CORT in feed (CORT), challenged with *C. perfringens* (*Cp*), or received both CORT and *C. perfringens* treatment (*Cp* + CORT). CORT treatment commenced at 11 days post-hatch (p.h.). Birds were orally administered 1-2 x 10^8 CFU *C. perfringens* on days 12 and 13 p.h., and necropsies were conducted on day 14 p.h. (A) *IL2,* (B) *IL1B,* (C) *IL17,* (D) *TGFB2,* (E) *iNOS,* (F) *TLR2A.* Vertical lines associated with histogram bars represent standard error of the means (n = 6).

<u>Chapter 6:</u> The administration of a complex microbiota to day-old chicks provides resistance to necrotic enteritis through the modulation of the intestinal microbiota, metabolome, and host responses.

6.1 Abstract

The objectives of this study were to establish a complex microbiota (CM) derived from the ceca of healthy adult broiler breeders in an anaerobic bioreactor system, administer the CM to day-old broiler chicks, measure the impacts of the CM on enteric bacterial community structure, and ascertain the degree to which and how the CM affects necrotic enteritis (NE). To induce NE, birds were administered in-feed corticosterone (CORT; 20 mg/kg) beginning at day 11 and subsequently challenged with *Clostridium perfringens* on days 12 and 13 post-hatch. Birds were evaluated on days 14 and 16 post-hatch. Bacterial diversity in the bioreactors was reduced relative to donor birds. Few viruses were detected in bioreactor samples and subsequently in birds administered the CM. Birds administered the CM exhibited a substantially higher α- and β-diversity of bacteria in their jejunums and ceca. Three taxa in the jejunum and 16 taxa in the ceca were conspicuously altered ± CM-treated birds co-challenged with *C. perfringens* and CORT. Specifically, butyrate-producing bacteria were increased in CM treated birds. Metabolite profiles in ceca digesta were altered in ± CM treated birds co-challenged with *C. perfringens* and CORT. There were 59 metabolites differentially affected following CM treatment, and the relative levels of short chain fatty acids, butyrate, valerate, and propionate, were decreased in diseased birds. Birds receiving the CM manifested significantly less necrotic lesions. Corticosterone administration decreased gene expression of *TLR2A*, *IL1B*, *CATH1*, and *MUC2B* in the jejunum 2 days post-inoculation (p.i.) with *C. perfringens*. Conversely, CM treatment promoted gene expression of *IL22*, *IL2*, and *IL17* 2 days p.i. with the pathogen indicating these birds were immunologically equipped to respond to pathogen challenge. Collectively, these results demonstrate that providing day-old birds with a complex mixture of bacteria prevented NE in broilers by increasing bacterial diversity and promoting positive immune responses.

6.2 Introduction

Fecal microbiota transplants (FMTs) have emerged as a therapeutic approach to treating disease in humans and is beginning to gain interest in agriculture. The process of a FMT involves the transfer of a complete microbial community that includes viruses, archaea, bacteria, fungi, protozoa, host cells, metabolites, and other debris [134]. Beneficial outcomes of FMTs can include restructuring microbial communities and increasing community diversity [274]. Furthermore, the introduction of novel microbes may stimulate the immune system in a manner that limits infection [274]. In therapeutic

applications, FMTs are thought to function by promoting positive microbe-microbe and microbe-host interactions that displace pathogens from microbial communities and reduce pathological outcomes [9].

Necrotic enteritis (NE) is a disease of the small intestine of poultry that is caused by *Clostridium perfringens* and results in significant production losses [216]. The administration of in-feed antibiotics has previously controlled NE, but reductions in antibiotic use have resulted in increased incidences of NE [275]. One antimicrobial alternative that has been widely explored is probiotics. The administration of probiotics has shown to limit NE through competitive exclusion, stimulating immune functions, and bacteriocin production [276]. *Lactobacillus* and *Bacillus* spp. are the most studied probiotics in poultry. *Lactobacillus johnsonii* has shown to enhance antibody levels in the ileum, increase $CD3^+$ cells in the lamina propria, augment digestive functions, reduce the expression of lipid synthesis and inflammatory genes, and decrease detection of *C. perfringens* [277-279]. A strain of *Bacillus subtilis* isolated from healthy chickens has shown to produce an antimicrobial compound effective against *C. perfringens* and has the advantage of being administered as an environmentally resistant spore [280]. The application of microbiota transplants has been considered in poultry in the context of enhancing production efficiency and determining if behavioural phenotypes can be passed through the microbiota [135-137]. However, the exploration of FMT in poultry has yet to be considered as an antimicrobial alternative to NE. Given the benefits that administering a single type of bacteria can have on modulating host responses to limit NE, the application of microbiota transplants as a method to prevent NE is warranted.

Poultry are often exposed to hygienic environments after hatching and this results in delayed exposure of microbes in comparison to birds that are raised in a wild environments [8]. Thus, the external application of a diverse set of microbes to birds early in life may expediate the development of the intestinal microbiota and confer positive health benefits. Probiotics in poultry focus on species that are primary colonizers of the small intestine, which is rational as NE impacts this region the greatest. However, the ceca are an important component of the intestinal tract as its microbes aid in digestion and promote immune development. Furthermore, commonly used probiotics, such as *Lactobacillus* spp., can facilitate cross-feeding to microbes in the ceca that utilize lactate to produce butyrate [281]. Therefore, the objective of this study was to inoculate day-old birds with a complex microbiota (CM) derived from the ceca of mature broiler breeders and determine its efficacy in preventing stress-induced NE later in life. My previous research developed a model of stress-induced NE that utilized dietary corticosterone (CORT) to mediate a stress response and promote the onset of NE. I hypothesize the introduction of cecal microbes will promote beneficial alterations to microbial community structure, increase diversity, and will modulate metabolites in the intestine that aid in disease resistance.

Furthermore, I anticipate that the application of a CM early in life will promote immune development that facilitates stronger immune responses when challenged with *C. perfringens* later in life.

6.3 Materials and Methods

6.3.1 Animal and biosafety approvals

The study was reviewed and approved before commencement by the Lethbridge Research and Development Centre (LeRDC) Animal Care Committee (Animal Use Protocol #1912). The study was executed in strict accordance with the Canadian Council on Animal Care Guidelines. In addition, approval to conduct research with *C. perfringens* was approved in advance by the LeRDC Biosafety and Biosecurity Committee. All of the requisite physical containment and operational practice requirements specified in the Public Health of Canada Canadian Biosafety Standard [282] for Containment Level 2 (CL2) laboratories and animal facilities were met.

6.3.2 Experimental design

The experiment was arranged as a complete randomized design with two levels of complex microbiota administration (± CM), two levels of CORT treatment (± CORT), and two levels of *C. perfringens* challenge (± *C. perfringens*) forming eight experimental groups (n=6 birds per group). Three birds from each treatment were euthanized and sample at 14- and 16-days post-hatch. The eight treatment were: (1) no CM, CORT, or *C. perfringens* treatment (Control); (2) *C. perfringens* challenge alone (*C. perfringens*); (3) CORT administration alone (CORT); (4) *C. perfringens* and CORT challenge (*C. perfringens* + CORT); (5) CM administration alone (CM); (6) CM and *C. perfringens* treatment (CM + *C. perfringens*); (7) CM and CORT treatment (CM + CORT); and (8) CM, CORT, and *C. perfringens* treatment (CM + *C. perfringens* + CORT).

6.3.3 Animals and husbandry

Ross 308 broiler eggs were obtained from a local hatchery (Lethbridge, AB, CA) and were incubated in a Brinsea Ovation 56 EX fully automatic digital egg incubator (Brinsea Products Inc., Titusville, FL) as previously described (see chapter 5). After hatch, arbitrarily chosen chicks were placed in pairs within individually ventilated cages (1862 cm^2 floor space; Techniplast, Montreal, QC) with sterile wood shavings for bedding. Birds were provided free access to water and food at all times. A starter diet was given for the first 10 days and a grower diet from day 11 until the end of the experiment (diet in Table 1, Chapter 5). Birds were maintained at 30°C for 2 days, 28°C for 2 days, then maintained at 26°C for the remained of the experiment on a 18 hr light: 6 hr dark cycle.

6.3.4 Complex microbiota generation

Generation of the CM was previously described in Low et al. [283]. Briefly, digesta from the ceca of six-month-old healthy male broiler breeders was harvested within 30 min of death. Ceca were ligated at the ileal-ceca junction to prevent infiltration of air into ceca, and excised from the intestinal tract. The ligated ceca were immediately transferred into an anaerobic chamber containing a nitrogen-predominant atmosphere (85:5:10% N_2:H_2:CO_2). Digesta was thoroughly mixed, transferred to tubes, and stored at -80°C until required. A continuous-flow mini-bioreactor system [284] was used to generate CM and standardize inoculum. Mini-bioreactors were situated within a Thermo Forma 1025 anaerobic chamber (Thermo Fisher Scientific Inc., Waltham, MA) containing the nitrogen-predominant gas atmosphere at 37°C. Cecal digesta was thawed on ice, and suspended in bioreactor medium consisting of 1 g/L tryptone, 2 g/L proteose peptone, 2 g/L yeast extract, 0.1 g/L arabinogalactan, 0.15 g/L maltose, 0.15 g/L D-cellobiose, 0.4 g/L sodium chloride, 5 mg/L hemin, 0.01 g/L magnesium sulfate, 0.01 g/L calcium chloride, 0.04 g/L potassium phosphate monobasic, 0.04 g/L potassium phosphate dibasic, and 2 mL/L Tween 80 at pH 6.8 [285]. The diluted feces was added to an individual bioreactor vessel at a final concentration of 25% w/v and allowed to incubate in the medium for one day without continuous flow of media. Each bioreactor vessel had an inflow and outflow tube that connected to a peristaltic pump that facilitated nutrient exchange. Pumps were set to an intake rate of 2 RPM (3.76 mL/hr) and an outflow rate of 4 RPM (7.52 mL/h). After 12 days, the CM was collected, and individual day-old chicks were gavaged with 1 mL of the CM or medium alone using a 18-gauge by 5-cm-long gavage needle. In addition, a 1 mL sample from each bioreactor was centrifuged at 13,000 x g for 5 min, the supernatant was removed, and the pellet stored at -80°C until processing for bacterial community analysis by 16S rRNA gene sequencing. Samples were also stored for virome analysis to ascertain quantities of viruses within the CM.

6.3.5 *Clostridium perfringens* inoculum preparation and inoculation of birds

Inoculum was prepared as described previously (see Chapter 5). Briefly, a starter culture of *C. perfringens* (CP1) was generated in heart infusion broth and incubated at 37°C within an anaerobic chamber containing the nitrogen-predominant atmosphere. The following day, 2.5 mL of the overnight culture was transferred to 50 mL fluid thioglycolate medium within the anaerobic chamber, and the culture was incubated at 37°C for 4 hr. At days 12 and 13 post-hatch, birds were orally inoculated with 1 mL of *C. perfringens* (1-2 x 10^8 CFU total) as described above for CM.

6.3.6 Corticosterone administration

Corticosterone was incorporated into the grower diet to attain a dose of 20 mg/kg. Corticosterone administration began at day 11 post-hatch and continued until the end of the experiment. Fresh feed was given to birds each morning and afternoon.

6.3.7 Collection of feces

Fresh feces was collected from birds 9 days after administration of the CM for virome analysis. Immediately after collection, individual fecal samples were snap frozen in liquid nitrogen, and stored at -80°C.

6.3.8 Animal euthanasia and sample collection

At defined endpoints, birds were anesthetized with isoflurane (5% isoflurane; 1 L O_2/min) and euthanized by cervical dislocation while under general anesthesia. The abdomen was opened, and blood was drawn directly from the heart upon cervical dislocation to collect serum for CORT quantification. The small intestine was aseptically removed and examined for disease progression and lesion scoring. Jejunum tissue was flash frozen in liquid nitrogen for metabolomic analysis, stored in RNAlater™ Stabilization Solution for quantification of mRNA, or placed in 10% neutral buffered formalin (Leica, Concord, ON) for histopathological examination. Digesta from the jejunum and ceca was removed using a sterile wooden splint, and snap frozen. With the exception of samples for histopathologic analysis, all samples were stored at -80°C until processed.

6.3.9 Lesion scoring and histopathology

The entire length of the small intestine (duodenum to ileal-cecal junction) was examined for gross lesions and scored 0-6 as described by Shojadoost et al. 2012 [114]. Samples for histopathologic examination were processed as describe previously (see Chapter 5). Briefly, samples were taken in the proximal jejunum region in all birds; lesions were not sampled. Jejunal tissue was fixed for a minimum 24 hr before being processed. Samples were embedded into paraffin blocks, sectioned (5 µm), deparaffinized with xylene, and stained with hematoxylin and eosin [193]. Sections were scored by a pathologist (V.F.B) blinded to treatments using a modified scoring system based on previously described methods [121, 234, 267]. In this regard, sections were graded from 0 to 4 for villus fusion, villus atrophy, mucosal necrosis, bacterial invasion, and lamina propria changes (Table 2). Total histologic scores were determined by calculating the sum of scores from all categories. Data were analyzed using the Kruskal-Wallis test with Dunn's test for multiple comparison (GraphPad Prism software; La Jolla, CA; version 9.1.2). P-values ≤ 0.050 were considered significant.

6.3.10 Quantification of *Clostridium perfringens*

DNA was extracted from jejunal digesta using a Qiagen QIAamp DNA Fast Stool Kit (Qiagen Inc.) with modifications (see Chapter 5). A standard curve of known copies of 16S rRNA gene DNA specific to *C. perfringens* was generated as previously described [101, 193]; the standard curve was generated using a 10-fold dilution series ranging from 10^7 copies to 10^1 copies of the *C. perfringens* 16S rRNA gene. Primers for *C. perfringens* were F: 5'-AAAGATGGCATCATCATTCAAC and R: 5'-TACCGTCATTATCTTCCCCAAA (Integrated DNA Technologies, Coralville, IA) [199]. Each reaction contained 10 µL Quantitect SYBR green master mix (Qiagen Inc.), 1 µL of each primer, 2 µL bovine serum albumin, 4 µL DNase-free water, and 2 µL of template DNA (10 ng/µL). Reactions conditions were: 95°C for 15 min; and 40 cycles of 95°C for 15 s, 55°C for 30 s, and 72°C for 30 s, followed by melt curve analysis from 55-95°C. A MxPro 3005P thermocycler (Agilent Technologies, Santa Clara, CA) was used to conduct qPCR analysis. Each reaction was run in duplicate, and the mean of the two observations was calculated. Data was log transformed to achieve normality and analyzed by three-way analysis of variance (ANOVA) with Tukey's multiple comparisons test (GraphPad Prism); experimental factors were CM, CORT, and *C. perfringens*. P-values ≤ 0.050 were considered significant.

6.3.11 Characterization of bacterial communities

Jejunum and cecal digesta DNA was extracted using Qiagen QIAamp DNA Fast Stool Kit (Qiagen Inc.) as described previously (see Chapter 5). Briefly, the V4 region of the 16S rRNA gene was amplified using a protocol developed by Kozich et al. [286]. The primers used were F-5'-GTGCCAGCMGCCGCGGTAA-3' and R-5'-GGACTACHVGGGTWTCTAAT-3' [286]. The PCR reactions contained: 12.5 µL of Paq5000 Hi Fidelity Taq Master Mix (Agilent Technologies Canada Inc.); 1 µL each of primer (10 µM; Integrated DNA Technologies); 5 µL (jejunum) or 2 µL (ceca) DNA; 5.5 µL (jejunum) or 8.5 µL (ceca) nuclease-free water (Qiagen Inc.). Reaction conditions were: 95°C for 2 min; 25 (ceca) or 30 (jejunum) cycles of 95°C for 20 sec, 55°C for 15 sec, and 72°C for 5 min; and 1 cycle at 72°C for 10 min. AMPure XP beads (Beckman Coulter Diagnostics, Brea, CA) were used to purify PCR amplicons. An Agilent High Sensitivity DNA chips were used on a Bioanalyzer 2100 (Agilent Technologies Canada Inc.) to check purified amplicons for quality and size. Amplicon were quantified using a Qubit 4 fluorometer (Thermo Fisher Science). DNA samples were normalized to 6 nM, pooled, denatured with NaOH, and diluted with HT1 (Illumina, San Diego, CA) to produce a 6 pM library for sequencing analysis. PhiX control DNA (25%) was added to the library as a sequencing control. The library was loaded onto a MiSeq Reagent Kit v2 500-cycle and run on an Illumina MiSeq platform (Illumina).

Quantitative Insights Into Microbial Ecology 2 (QIIME2™, version 2021.2 [194]) was used to execute sequencing analysis. DADA2 was used to filter low quality reads (quality score <20) and trim sequences. Forward reads were grouped into exact amplicon sequence variants (ASVs) and taxonomy was classified using the SILVA bacteria reference database (release 138) [195]. Low count reads, mitochondrial sequences, and chloroplast sequences were filtered out. Sampling depth was 11,012 and 85,403 reads for the jejunum and ceca digesta, respectively. There was no difference among the two sample time points and analysis was averaged over the two times. Core metrics phylogeny analysis was performed in QIIME2 to obtain α- and β-diversity. Alpha diversity was analyzed in QIIME2 by pairwise comparisons of Kruskal-Wallis test. Beta diversity was analyzed by pairwise permutational multivariate analysis of variance (PERMANOVA) in QIIME2 [196]. A Bejamini and Hochberg correction was applied to both α- and β-diversity tests when corrected P-values were ≤ 0.050. The R package for ANOVA-like differential expression 2 (ALDEx2) plugin was used in QIIME2 to differentiate significant taxa (q ≤ 0.100) between the two CM treatment birds (±) that were not challenged with *C. perfringens* or CORT, and challenged with *C. perfringens* alone, CORT alone, or both *C. perfringens* and CORT [197]. Percent abundance of taxa (*C. perfringens* and *Lactobacillus* in the jejunum; *Megamonas* and *Bacteroides* in the ceca) was normalized, and a three-way ANOVA was applied with a multiple comparison Tukey's significant difference test (GraphPad Prism). P-values ≤ 0.050 were considered significant.

6.3.12 Characterization of the virome

Characterization of the virome in fecal samples obtained from birds (2 g) and from CM bioreactors (2 mL) were determined by metagenomics analysis. Samples were homogenized in 20% PBS and centrifuged at 16,000 × *g* at 4°C for 5 min. After centrifugation, supernatants were successively filtrated through 0.45 µm and 0.22 µm filters. A 140 µL subsample of each filtrate was treated with 2 µL of RNase A (Thermo Fisher Science), incubated for 15 min at 37°C, and inactivated with 2 µL of RiboLock RNase inhibitor (Thermo Fisher Science) for 3 min at 4°C. DNase I (2.5 µL; Sigma-Aldrich, St. Louis, MO) was added to each sample, incubated 45 min at 37°C and heat inactivated for 10 min at 65°C. Genetic material was extracted using a QIAamp Viral RNA mini kit (Qiagen Inc.) following the protocol recommended by the manufacturer, with the exception that the RNA carrier was omitted.
Genetic materials were reverse transcribed using a Superscript III (Invitrogen, Mississauga, ON) and 40 pmol of primer A (5'-GTTTCCCAGTCACGATA-(N9)-3') [287] according to the manufacturer's recommendations. A second-strand synthesis was performed by adding 10 µL of a Sequenase mix (0.3 µL of 1:8 diluted Sequenase) (Sigma-Aldrich) using the following thermal conditions: a temperature ramping from 10 to 37°C for 8 min, holding at 37°C for 8 min, heating to 94°C for 2 min, and cooling to

10°C. PCR amplification was then performed in a 40-µL volume containing 10 µL of the Sequenase reaction with 100 pmol of primer B (5'-GTTTCCCAGTCACGATA-3') and 1 µL of AccuTaq DNA polymerase (Sigma-Aldrich) following the manufacturer's recommendations. All samples were purified using a QIAquick PCR purification kit (Qiagen) and quantified using a Qubit dsDNA BR Assay Kit (Thermo Fisher Science). Libraries were prepared using the purified amplicons and a Illumina DNA prep Kit (Illumina) and Nextera DNA CD 24 indexes. Then paired-end sequenced using a MiSeq sequencing platform (Illumina) with a 300-cycle MiSeq Reagent Kit v3 (Illumina). Metgenomic analysis were performed as described by Natel-Fortier et al. [288].

6.3.13 Characterization of the metabolome

A ^{1}H-Nuclear Magnetic Resonance (NMR) spectrometer was used to characterize metabolites in samples as described previously [289]. Briefly, jejunal digesta, jejunal tissue, and cecal digesta samples (150 mg) were suspended in metabolomics buffer (0.125 M KH_2PO_4, 0.5 M K_2HPO_4, 0.00375 M NaN_3, and 0.375 M KF; pH 7.4). Samples were homogenized with one 6-mm-diameter steel bead for 10 min using a Qiagen Tissue Lyser LT (Qiagen Inc.) operated at 50 Hz. Homogenized samples were centrifuged for 5 min at 14,000 x g. The supernatant of each sample was passed through a 3000 MWCO Amicon Ultra-0.5 filter (Millipore Sigma, Oakville, ON, CA) by centrifuging at 14,000 x g for 30 min at 4°C; filters were rinsed with Millipore water ten times prior to use. For each filtrate, 360 µL was mixed with 200 µL metabolomics buffer and 140 µL deuterium oxide containing 0.05% v/v trimethylsilylpropanoic acid (TSP) to yield a final volume of 700 µL. TSP was used as a chemical shift reference for ^{1}H-NMR spectroscopy. To prepare the solution for spectroscopy, it was vortexed, centrifuged at 12,000 x g for 5 min at 4°C, and a 550 µL aliquot of the supernatant was loaded in a 5 mm NMR tube. Samples were run on a 700 MHz Bruker Avance III HD NMR spectrometer (Bruker, Milton, ON, CA) for spectral acquisition, and data collection and spectral processing was conducted as described previously [289]. MATLAB (MathWorks, Natick, MA) was used to align spectral peaks through Recursive Segment Wise Peak Alignment [161] and binning with Dynamic Adaptive Binning [162]. Data was normalized to the total metabolome, excluding the region containing the water peak, and pareto scaled.

MATLAB (Math Works) and the Metaboanalyst R package was used for metabolomics analysis [290]. To determine which metabolites were significantly altered between treatments, spectral bins were subjected to univariate and multivariate analysis. The univariate measure were calculated using a decision tree algorithm, as previously described by Goodpaster et al [164]. The multivariate tests utilized the Variable Importance Analysis based on random Variable Combination (VIAVC) algorithm. This combines both Partial Least Squares Discriminant Analysis (PLS-DA) and the area under the Receiver

Operating Characteristics (ROC) curve to synergistically determine the best subset of metabolites for group classifications [165]. All P-values obtained from analysis were Bonferroni-Holm corrected for multiple comparisons. MATLAB was used to calculate the percent difference of bins between treatments. Metaboanalyst R package was used to facilitate orthogonal partial least squares discriminant analysis (OPLS-DA). Chenomx 8.2 NMR Suite (Chenomx INC., Edmonton, AB, CA) was used to identify metabolites. MetaboAnalyst 5.0 was used to determine metabolic pathways that were associated with distinguished metabolites. P-values ≤ 0.050 were considered significant.

6.3.14 Determination of relative mRNA gene quantities

Methods previously described in Chapter 5 were followed. Briefly, RNA was extracted from jejunal tissue using RNeasy Plus Mini Kit (Qiagen Inc.) and accessed for quality and quantity using an Agilent Bioanalyzer (Agilent Technologies). RNA (1 µg) was reverse transcribed to cDNA using a QuantiTect reverse transcription kit (Qiagen Inc.). A MxPro 3005P thermocycler (Agilent Technologies) was used to perform PCR reactions. Each reaction contained 5 µL Quantitect SYBR green master mix (Qiagen Inc.), 0.5 µL of each primer (10 µM), 3 µL of RNase-free water, and 1 µL of cDNA. Reaction conditions were: 95°C for 15 min; 40 cycles of 95°C for 15 sec, 55-58°C for 30 sec, 72°C for 30 sec; and a melt curve analysis from 55 to 95°C. Unless cited otherwise, primers were designed using NCBI primer BLAST (Table 6.1). Reactions were run in duplicate and the average ct values were used to calculate mRNA concentrations relative to two reference genes (β-actin and GAPDH) using qBase+ software (Biogazelle, Gent, Belgium) [158]. Data was log transformed to achieve normality and analyzed in GraphPad Prism (version 9.1.2) by three-way ANOVA with Tukey's multiple comparisons test where the three experimental factors were CM, CORT, and *C. perfringens*. P-values ≤ 0.050 were considered significant.

6.3.15 Quantification of corticosterone serum concentrations

Serum CORT concentration was measured by enzyme-linked immunosorbent assay (Cayman Chemical Company, Ann Arbor, MI) following the manufacturer's recommendations. Concentrations were computed using logistic regression model, and a three-way ANOVA (GraphPad Prism) was conducted with Tukey's multiple comparison test where the three experimental factors were CM, CORT, and *C. perfringens*; P-values ≤ 0.050 were considered significant.

6.4 Results

6.4.1 The structure of cecal bacterial communities in bioreactors differed from donor birds

Taxonomic classification at the genus level was used to ascertain bacterial richness in cecal donor digesta and within bioreactors (Figure 6.1A-B). Cecal digesta contained 128 genera, whereas bioreactor matrix contained 52 genera. Bacteria within the genera, *Bacteroides*, *Rikenellaceae*,

Phascolarctobacterium, and *Prevotellaceae* were less abundant in the bioreactors than in cecal digesta. Despite the decrease in *Bacteroides and Phascolarctobacterium* in the bioreactors, these taxa were observed to be predominate in the cecal of birds administered CM (Figure 6.1C). *Megamonas*, a taxon that was enriched in the bioreactors relative to cecal inoculum, was also a prominent colonizer of the ceca of birds administered CM.

6.4.2 Limited numbers of viruses were detected in bioreactor samples and in birds administered the complex microbiota

Next-generation sequence analysis of the virome revealed that viral sequences (family level of resolution) within the bioreactor matrix and in feces from birds ± administration of CM accounted for < 0.025% of sequence counts, which suggests that viral loads in bioreactors and feces were exceptionally low (Figure 6.1D). Notably, many of the prominent viruses detected have microorganisms as their primary host (e.g. *Siphoviridae*, *Myoviridae*, *Mimiviridae*). Viral families that contain members that are potential pathogens were *Reoviridae*, *Retroviridae*, *Poxviridae*, *Herpesviridae*, but were observed at low relative levels (< 0.010%).

6.4.3 The enteric bacterial community structure was altered in birds treated with *Clostridium perfringens* and corticosterone following complex microbiota administration

The administration of the CM resulted in larger numbers of ASVs in the jejunum (31 without CM; 61 with CM) and in ceca (58 without CM; 109 with CM). Alpha diversity (Faith's phylogenetic diversity) was higher in the jejunal digesta ($P = 0.016$) and cecal digesta ($P = 0.004$; Figure 6.2A) of birds administered the CM. Moreover, the structure of the bacterial communities (Unweighted UniFrac) in jejunal digesta ($P = 0.004$) and cecal digesta ($P = 0.006$) of birds receiving the CM were conspicuously altered (Figure 6.2B). The observed change in α- and β-diversity was associated with a conspicuous change in the relative abundance of multiple bacterial taxa in the jejunum and ceca (Figure 6.2C-D). The relative abundance of *C. perfringens* and *Lactobacillus* spp. ASVs was lower ($P \leq 0.107$) in the jejunum of birds administered CM (Figure 6.2E). The relative abundance of *Megamonas* and *Bacteroides* were higher ($P \leq 0.036$) in the ceca of birds administered CM (Figure 6.2F). ALDEx2 analysis was applied to identify taxa that were differentially affected by the administration of the CM with *C. perfringens* and CORT co-challenge (Figure 6.3). In the jejunum, *Megamonas* and *Olsenella* were more abundant in CM treated birds, while *Lachnoclostridium* was more abundant in birds not administered CM. There were 16 differentially altered taxa in the ceca. Nine taxa were increased in birds that did not receive the CM. Seven taxa were more abundant with CM administration with some being notable SCFA producers (*Megamonas*, *Megasphaera*, and *Phascplarctobacterium*).

6.4.4 The metabolome of cecal digesta was altered in birds treated with *Clostridium perfringens* and corticosterone following complex microbiota administration

Water-soluble metabolites were extracted from jejunum digesta, jejunum tissue, and cecal digesta and subjected to ^{1}H-NMR spectroscopy to identify changes in the metabolome associated with the CM administration. In total, 432, 550, and 178 metabolite bins were detected in the jejunal digesta, jejunal tissue, and cecal digesta, respectively. Supervised OPLS-DA separation showed no significant changes in the metabolome due to CM administration in either jejunal digesta (Figure 6.4A; $P = 0.274$, $Q^2 = 0.018$, $R^2 = 0.734$) or jejunal tissue (Figure 6.4B; $P = 0.685$, $Q^2 = -0.421$, $R^2 = 0.989$). However, three metabolites in jejunum digesta and seven metabolites in jejunum tissue of birds administered the CM were altered (Figure 6.4C-D). In jejunal digesta, citrate, dimethylamine, and 4,5-dihydroorotic acid were increased in birds not administered the CM. In jejunal tissue, three metabolites (fructose, glycerophosphocholine, NADH) were increased, while 4 metabolites (lysine, proline, uracil, and cytidine monophosphate) were decreased in birds not administered the CM. Supervised OPLS-DA separation showed that the CM treatment altered metabolite profiles in cecal digesta (Figure 6.5A; $P = 0.002$, $Q^2 = 0.873$, $R^2 = 0.994$). In this regard, 59 metabolites were differentiated affected because of the administration of the CM (Figure 6.5B). Metabolites involved in amino acid metabolism and short-chain fatty acids (SCFA) were decreased in birds that did not receive the CM and were the most diseased.

6.4.5 Complex microbiota administration reduced gross pathologies

Only birds inoculated with *C. perfringens* exhibited necrotic lesions (Figure 6.6A). Birds treated with *C. perfringens and* CORT had higher ($P \leq 0.036$) necrotic lesions scores in comparison to all other treatments (Figure 6.6B). Likewise, densities of *C. perfringens* were highest ($P \leq 0.017$) in *C. perfringens and* CORT treatment birds in comparison to all other treatments (Figure 6.6C). Although there was no significant difference ($P \geq 0.240$) among treatments, cumulative total scores for the CM +, *C. perfringens* +, CORT + treatment birds trended lower than for *C. perfringens* + and CORT + treatment birds not receiving the CM, which was primarily associated with reduced degrees of villus atrophy, bacterial invasion, and mucosal necrosis (Figure 6.6D-E).

6.4.6 Corticosterone and *Clostridium perfringens* promoted disease by modulating intestinal responses

To determine how stress influenced the onset of NE, immune and mucus gene responses in the intestine were evaluated by measuring relative mRNA gene quantities of *IL1B*, *IL17*, *TGFB2*, *TLR2A*, *MUC2B*, and *MUC5AC* at 2 days p.i. with *C. perfringens* (Figure 6.7). The administration of CORT had no effect ($P \geq 0.141$) on gene expression at 4 days p.i. with *C. perfringens*. In birds administered CORT,

relative mRNA quantities of *IL1B* (P = 0.045), *TLR2A* (P = 0.004), and *MUC2B* (P = 0.002) decreased in comparison to birds not administered CORT. Birds co-challenged with *C. perfringens* and CORT exhibited decreased *IL17* (P = 0.038) and MUC5AC (P = 0.044) mRNA quantities. Birds inoculated with *C. perfringens* had increased *TGFB2* (P = 0.045).

6.4.7 Tight junction proteins and antimicrobial peptides were altered by experimental treatments

Relative mRNA gene quantities of *CLD3*, *TJP1*, *AvBD6*, and *CATH1* were examined at 2 and 4 days p.i. with *C. perfringens* to determine if barrier and epithelial functions were altered by the experimental treatments. Increased *CLD3* mRNA was observed in birds administered CORT at 2 (P < 0.001) and 4 (P = 0.001) days p.i. with *C. perfringens* (Figure 6.8A). At 4 days p.i., birds challenged with *C. perfringens* exhibited increased (P = 0.040) quantities of *CLD3* mRNA. Additionally, birds administered the CM and subsequently challenged with *C. perfringens* showed increased *CLD3* (P = 0.002) and *TJP1* (P = 0.050) mRNA (Figure 6.8B-D). Birds administered the CM tended to show decreased (P = 0.058) *AvBD6* mRNA quantity at 2 days p.i. (Figure 6.8E). At 2 days p.i., birds co-challenged with CORT and *C. perfringens* exhibited decreased (P=0.030) *CATH1* mRNA quantities (Figure 6.8G). Conversely, at 4 days p.i., birds co-challenged with *C. perfringens* and CORT showed increased expression of *AvBD6* (P = 0.010) and *CATH1* (P = 0.009) mRNA (Figure 6.8F+H).

6.4.8 Complex microbiota promotes disease responses in the jejunum

Complex microbiota treatment altered mRNA gene quantities of *IL22*, *IL2*, *IL17*, and *MUC2B*. In this regard, birds administered the CM showed increased *IL22* (P = 0.007), *IL2* (P = 0.012), and *IL17* (P = 0.006) mRNA expression at 2 days p.i. with *C. perfringens* (Figure 6.9A;C;E). At 4 days p.i. with *C. perfringens*, birds administered CM displayed increased quantities of *IL2* (P = 0.049) and *MUC2B* (P = 0.003) mRNA (Figure 6.9B;D).

6.4.9 Complex microbiota decreases serum corticosterone

Chickens administered CORT exhibited higher (P < 0.001) serum concentration of CORT (Figure 6.10). Serum CORT concentrations were decreased (P = 0.050) in birds administered CM independent of *C. perfringens* infection.

6.5 Discussion

There are many possible mechanisms by which introduced enteric bacteria can confer beneficial impacts on health, immune function, and disease resistance. Two mechanistic concepts that are often explored by researchers are: (1) microbe-microbe interactions; and (2) microbe-host interactions [105]. Microbe-microbe interactions can include microbes competing for nutrient resources and/or limiting the growth of other bacteria through the production of inhibitory molecules (e.g. bacteriocins) [106].

Microbe-host interactions include microbes stimulating the mucosal immune system, protecting barrier function, and promoting host defence responses in the intestine [106]. Few studies conducted to date in poultry have explored how the exogenous administration of bacteria may impact bird health and NE. Some research has shown the probiotic, *L. johnsonii,* can affect immune responses in the intestine and alter lipid metabolism in subclinical NE [277, 278, 291]. In the current study, the amelioration of NE in the jejunum of broilers following the administration of a CM was observed; this included decreased necrotic lesions and reduced densities of *C. perfringens*. The reduction of NE through microbiota transplantation has not previously been explored in chickens to my knowledge, although, competitive exclusion and probiotic strategies have been previously studied [277, 292]. A competitive exclusion mixture of lactic acid bacteria has been shown to decrease lesions of *C. perfringens* challenged birds [292]. *L. johnsonii* BS15 administration showed to alleviate the impacts of subclinical NE on bird weight gain and reduce damage to villi in the ileum [277]. Importantly, limited research in poultry has focused on deciphering how probiotics, or microbiota transplants, can provide disease resistance. Thus, a salient objective of the current study was to explore how a transplant of a complex bacterial community to day-old chicks ameliorates NE by evaluating the intestinal microbiota, metabolome, and host responses (i.e. intestinal barrier function, host defence stimulation, and impacts on mucins).

Changes to bacterial communities in birds developing NE have been previously described [191, 218]. However, it remains unclear whether a change in the intestinal microbiota is a predisposing factor to NE, or whether it is the result of an infection [293]. Some predisposing factors to NE, such as a diet containing fishmeal, fumonisins, and/or *Eimeria* spp. have been shown to alter the intestinal microbiota [293, 294]. Likewise, my research has previously shown that CORT administration decreased α-diversity and altered community structure, and concomitantly increased densities of *C. perfringens* in the small intestine of chickens [101]. Production birds lack significant maternal exposure to microbes, and I hypothesized that the enhancement of bacterial richness and diversity could aid in pathogen resistance. A study that examined the impact of maternal exposure to chicks for 24 hr showed this interaction could provide an important source of *Bacteroidetes* and *Actinobacteria* [295]. Thus, an advantage to microbiota transplantation is the opportunity to increase the diversity of commensal bacteria, which may confer colonization resistance, including competition for nutrient niches [9]. In this regard, the present study showed a conspicuous and persistent increase in α- and β-diversity in broilers inoculated with the CM. Notably, this study identified an increase in bacterial richness in both the jejunum and ceca of birds administered CM. Additionally, birds co-challenged with CORT and *C. perfringens* displayed different compositions of bacteria with and without the CM treatment. The increased diversity of

potentially beneficial bacteria following microbiota transplantation has been linked to ensuring disease resistance [9].

Specific changes to microbes during NE have centered around the disturbances to lactic acid producing bacteria and short-chain fatty acid (SCFA) fermenting bacteria [293]. Fishmeal diets and NE can alter the distribution of *Lactobacillus* species [191, 294]. For example, in the ceca of *C. perfringens* infected birds, quantities of *L. johnsonii* and *L. fermentum* decreased, whereas quantities of *L. crispatus*, *L. pontis*, *L.ultunese*, and *L. salivarius* increased [191]. In the current study, *L. salivarius* was a bacterium that was introduced with the CM administration, and it increased in abundance in birds that were administered CORT and challenged with *C. perfringens*. The observed increase in abundance of *Lactobacillus* corresponded with a reduction in *C. perfringens* detection as determined by both Illumina sequencing and taxon-specific qPCR. Lactic acid bacteria may have played a role in reducing colonization by *C. perfringens* in the jejunum of CM treated birds through several mechanisms. Firstly, inhibitory compounds produced by *Lactobacillus* could have reduced the growth of *C. perfringens*. Reuterin and nisin, which are compounds produced by *Lactobacillus*, have been shown to inhibit the growth of vegetative cells and inhibit the germination of endospores of *C. perfringens* [296]. Secondly, a microbial cross-feeding interaction may have occurred. Lactate produced by *Lactobacillus* spp. can be metabolized by butyrate-producing bacteria, such as *Megamonas hypermegale* [297]. In the current study, *M. hypermegale* was observed to be differentially abundant in birds that were administered the CM and remained healthy. Thirdly, *Lactobacillus*, along with other bacteria introduced with the CM, may have modulated mucosal responses that resulted in alterations to immune and barrier function that antagonized colonization by *C. perfringens* [293, 298].

The ceca, and its microbiota, provides many benefits to chicken health. Some recognized functions of the ceca include nitrogen cycling, fermentation of sugars, antibody production, and the provision of essential amino acids to the host [299, 300]. Numerous alterations to the ceca microbiota and metabolome were observed in the CM treated birds that were administered CORT and challenged with *C. perfringens* in the current study. Notably, many metabolites involved in the metabolism of amino acids were decreased in birds in which NE was manifested (i.e. chicks that did not receive CM). Additionally, birds that did not receive the CM showed a relative decrease in propionate, butyrate, and valerate, which are prominent SCFA produced in the ceca of birds [289]. Alterations to SCFA production in the ceca of the CM treated birds may be one way in which the ceca microbiota was able to aid in disease resistance in the small intestine. In this regard, butyrate has been shown to act on signalling of enteroendocrine cells that leads to the release of glucagon-like peptide 2 into the bloodstream [293].

Systemic transport of this peptide can result in the activation of nerve cells in the small intestine to release cytokines that enhance the expression of barrier function proteins [301]. These results indicate that CM administration with *C. perfringens* infection could induce the expression of *CLD3* and *TJP1* in the small intestine, corresponding with higher SCFA levels observed in ceca.

Reductions in butyrate-producing bacteria have been demonstrated in chickens fed a diet amended with fishmeal and/or in birds infected with *C. perfringens* [191, 294]. In the ceca of birds not administered the CM, a decrease in abundance of *M. hypermegale* and *M. stantonii* was observed, both of which are bacteria that ferment carbohydrates into SCFA [297, 302]. Although the densities of these two bacterial species were decreased in birds with NE, an increase in the abundance of *Anaerostipes butyraticus* was observed, and this bacterium is also a known producer of butyrate [303]. It is known that some SCFAs (e.g. butyrate) can provide a host with energy, and also can be involved in the regulation of glucose and lipid metabolism [304]. It remains unclear whether the birds with NE in the current study exhibited a relative decrease in SCFAs due to less production by the microbiota, or as a result of rapid uptake by the host (e.g. by enterocytes) for energy demands.

The administration of CORT was observed to modulate immune responses in broiler chickens in the current study, and this may have facilitated the onset of NE. Similarly to a previous study conducted in a layer breed [193], the present study observed a decrease in the concentration of *TLR2A* mRNA in broiler chickens administered CORT. This may indicate that downstream inflammatory responses were reduced in birds administered CORT [193]. Correspondingly, a decrease in quantities of *IL1B* mRNA in birds administered CORT was observed, which is a downstream pathway component to TLR signalling [305]. A first line of defence against pathogens includes the innate immune system, where TLR signalling activates the transcription factors nuclear factor (NF)-κB and activating protein (AP)-1, resulting in the production of pro-inflammatory cytokines [305]. Notably, CORT can block pro-inflammatory responses through repressing NFκB and AP-1 [12, 13]. This may be one mechanism in which CORT administration reduced inflammatory responses in the current study. The current study showed a decrease in *IL17* mRNA in birds administered CORT and challenged with *C. perfringens*, but were not treated with the CM. Host defences in response to microorganisms are partly mediated through IL17 in the intestine, although knowledge gaps with respect to Th17 responses in chickens exist [306]. Functions of IL17 include inducing the expression of pro-inflammatory cytokines and chemokines [306]. Collaborative induction of pro-inflammatory responses by *IL17* can form a positive feedback loop that enhances the effects of IL17 [307]. Thus, it is possible that the repressive actions on CORT could rupture this feedback loop and result in decreased expression of IL17 in birds co-challenged with *C. perfringens* and CORT.

Evidence of a disruption to intestinal barrier defences were observed in the jejunum of broilers subjected to physiological stress incited by CORT. Mucus provides a physical barrier to limit microorganisms access to host cells [87]. In the present study, a decrease in *MUC2B* and *MUC5AC* mRNA in birds administered CORT was observed, although the expression of *MUC5AC* mRNA was only reduced in birds also challenged with *C. perfringens*. The production of mucins can be modulated by both the microbiota and immune responses [308, 309]. Thus, the observed decrease in *MUC2B* and *MUC5AC* mRNA expression may have corresponded to CORT-mediated suppression of inflammatory responses. This agrees with a study that demonstrated that dexamethasone exposure decreased mRNA expression of *MUC2* and *MUC5AC in vitro* [88]. My previous research has demonstrated that *C. perfringens* infection can modify the composition of mucus glycans [193]. Research to investigate the impacts that *C. perfringens* has on host mucins is warranted, as *MUC5AC* expression was only altered in the presence of *C. perfringens*. This study demonstrated that co-challenging birds with *C. perfringens* and CORT modulated the expression of *CATH1* and *AvBD6*. Notably, these two genes showed similar expression pattern, suggesting that they are regulated in a similar manner. Corticosterone administration decreased *CATH1* after 3 days of CORT administration, and this effect was exasperated by *C. perfringens* challenge. It has been demonstrated that *CATH1* possesses both antimicrobial and immunomodulatory properties [310]. Chicken *CATH1* has been shown to recruit neutrophils and modulate macrophage responses in a murine model [310]. It is possible, the initial decrease in *CATH1* coincided with CORT suppression to immune defences and this is consistent with other studies in mice [311]. At four days p.i. of *C. perfringens*, *CATH1* and *AvBD6* increased in birds co-challenged with *C. perfringens* and CORT. Increased expression may have been an attempt to limit *C. perfringens* infection as *AvBD6* has been previously seen to increase in the jejunum of Ross broilers with NE [312]. Alternatively, increases in host defence peptides could indicate their involvement in late onset immune responses to *C. perfringens* infection. This warrants examination.

Commensal bacteria promote the development of mucosal responses that limit pathogenic infections. The administration of the CM resulted in the modulation of epithelial responses that may have coincided with resistance to NE. This study showed increased *CLD3* expression with CORT treatment, which is consistent with my previous findings in layers [193]. However, it remains unclear whether a CORT-mediated increase in *CLD3* expression is indicative of enhanced or reduced barrier function, as increased expression of this protein has been reported for both scenarios [90, 244]. This study observed increased expression of *CLD3*, *TJP1*, and *MUC2B* mRNA following the CM treatment in birds infected with *C. perfringens* and this may indicate enhanced barrier function. Additionally, this may

denote healing responses post-infection were promoted in the CM treated birds. Increased fluorescein isothiocyanate dextran was detected in the serum of birds with NE, which indicates barrier function had been compromised [313]. It is not entirely clear if this effect was due to *C. perfringens* alone or whether it was a synergistic impact with co-incitant agents, as Latorre et al. [313] utilized *Salmonella enterica* serovar Typhimurium and *Eimeria maxima* to incite disease. Nonetheless, the upregulation of genes encoding proteins implicated in intestinal barrier function observed in the current study may coincide with the clearance of *C. perfringens* infection, which was enhanced in the CM treated birds.

In the current study, increased expression of *IL2*, *IL22*, and *IL17* mRNA 2 days after inoculation with *C. perfringens* was observed. Additionally, the CM treatment further elevated expression of *IL2* at 4 days p.i. with the pathogen. Although these cytokines are involved in inflammation, they are important mediators in mounting an immune response and limiting infection [314]. Thus, the elevation of these cytokines is consistent with the enhancement of mucosal immune development. Populations of CD3+ T-cells are observed throughout the intestinal tract of the GALT in newly hatched chicks; however, functional development of the GALT occurs in two stages post-hatch [315]. The first stage of development occurs at 4 days post-hatch, where T-cell maturation was associated with increases in *IL2* and *IFNγ* mRNA expression [315]. A second wave of maturation occurs during the second week post-hatch. At this time, prominent expression of *IL2* is observed in the jejunum, and this may coincide with maturation of lamina propria compartment [315]. It is unknown which cell types were expressing *IL2*, *IL22*, and *IL17* in birds treated with the CM. It is possible that the exposure to the CM shortly after hatch primed the GALT in a manner that promoted stronger functionality at 2 weeks post hatch, which is the time point of sampling in the current study. Thus, elevated expression of *IL2*, *IL22*, and *IL17* that was observed in the present study may be indicative of enhanced immune competence in birds administered the CM. This is consistent with an enhanced ability of these birds to combat infection and subsequent manifestation of disease.

An unanticipated finding from the current study was the observation of reduced concentrations of free corticosterone in the serum of birds administered CORT and CM in comparison to birds administered CORT without CM. Although the study was not design to elucidate why this occurred, several explanations are plausible. One possibility is that microbes introduced in the CM metabolized the CORT, thus leaving less CORT to enter systemic circulation. The breakdown of prednisolone has been demonstrated in simulated human colonic fluid [316]. Metabolites produced by the microbiota may have also influenced CORT concentrations. For example, mice supplemented with SCFA and exposed to chronic stress showed an acute decrease in CORT following a forced swim test [317]. Additionally, SCFA

treatment was associated with a decrease in the expression of corticotrophin-releasing factor receptor 1 in the colons of mice [317]. Another study demonstrated administration of *Lactobacillus farciminis* to rats was associated with an ensuing decrease in CORT concentrations when subjected to partial restraint stress [318]. This corresponded to the ability of *L. farciminis* to prevent stress-induced intestine permeability [318]. These observations from research with mammals demonstrate some possible mechanisms by which the microbiota may be involved in modulating stress responses, which requires validation in chickens.

In the current study, a bioreactor system to generate a CM (i.e. from healthy adult donor chickens) was utilized. A primary reason for selecting the bioreactor system to generate the CM was for the standardization of the bacterial community that was administered to day-old chicks. It is noteworthy, that although the bacterial community within the bioreactors was diverse and contained many obligate anaerobic taxa, both the α- and β-diversity of the bacterial community within the reactors was altered relative to the donor birds. Never-the-less, that the administration of the bioreactor-propagated CM was observed to ameliorate NE in broilers, coupled with the reduce diversity of CM from the bioreactors relative to the donor birds suggests that a core microbiota is required to confer colonization resistance. A major limitation of microbial transplants is the inadvertent transfer of pathogens from the donor, including important viral pathogens [134]. This has stimulated research to identify core taxa within the community that confer colonization resistance in treatment of human diseases using fecal transplants [319], and the administration of a defined bacterial consortium as an alternative to fecal or digesta transplants [320]. These findings suggest that similar research is warranted for application against chicken enteric diseases. As the inadvertent transfer of viral pathogens in the CM was a concern, the virome was examined for the bioreactor CM, and of birds receiving the CM relative to birds not receiving the CM. Unexpectedly, the number of viral reads were exceptionally low, which is indicative of low viral abundance within the bioreactor CM, and subsequently in the broiler chicks administered the CM. For comparison, my research lab has observed viral read proportions ranging from 1 to 12% from piglet feces, and 1% from air in pig barns (unpublished). Moreover, many of the prominent viral families detected in the bioreactor matrix and subsequently in the feces of birds contain viruses whose primary host is microorganisms (e.g. *Siphoviridae*, *Reoviridae*, *Myoviridae*, and *Mimiviridae*). Given the ability of ability of the bioreactor generated CM to ameliorate NE, ascertaining the impact of propagating CM in bioreactor systems on avian viral pathogen persistence is justifies further examination. Additionally, research to identify core compendium of bacteria that confers colonization resistance warrants investigation.

In summary, it was observed that the administration of a CM to day-old chicks prevented the onset of stress-induced NE. Increased α-diversity and altered β-diversity of bacterial communities in both the jejunum and ceca of the CM-treated birds was observed. Furthermore, the metabolome of cecal digesta, and to a lesser extent of jejunal digesta and tissue, showed a significant number of altered metabolites in birds administered the CM suggesting that the cecal microbiota imparts a systemic influence on disease resistance. It was also observed that stress promoted the onset of disease via the impairment of immune and barrier function. However, the administration of a CM counteracted these impacts by promoting mucosal immune competence. Collectively, the results demonstrated the importance of exposing young birds to a diverse set of microbes after hatch. Artificially increasing microbial diversity early in life may aid in the promotion of positive microbe-microbe and microbe-host interactions that promote immune development and resistance to important diseases, including NE.

6.6 Tables and Figures

Table 6.1 List of primer sequences for relative mRNA quantification.

Name				Abbreviation	Sequence 5' to 3'
Name	**Abbreviation**	**Forward**		**Annealling Temperature (°C)**	**Source**
Interleukin 1β	*IL1B*	TGCCTGCAGAAGAAGCCTCG	**Reverse**		
Interleukin 17A	*IL17A*	AGATGCTGGATGCCTAACCC	CTCCGCAGCAGTTTGGTCAT	58	[193]
Interleukin 2	*IL2*	TAACTGGGACACTGCCATGA	GTGGTCCTCATCGATCCTGTAA	58	Chapter 5
Interleukin 22	*IL22*	GGAATCGCACCTACACCTTG	GATAGAGATGCTCCATAAGCTGT	56	[193]
Transforming Growth Factor β2	*TGFB2*	CCATCTACAACAGCACCAGGG	GCGGTTGTTCTCCCTGATGT	58	This Study
Toll-like Receptor 2A	*TLR2A*	CAGCACAAGAGGCGTTCA	TAGCTTGGTGGGATGGCATTTTC	58	[193]
Mucin 2B	*MUC2B*	ATTGTGGTAACACCAACATTCATC	AACATTTTGGTGTAGCTGAGATG	56	[193]
Mucin 5AC	*MUC5AC*	TCCACCAGCTTCCAAATCCC	CTTTATAATGTCAGCACCAACTTCTC	56	[321]
Cathelicidin 1	*CATH1*	GCTGTGGACTCCTACAACCAAC	GGGGTTGCCAGCCTTTACTT	58	[193]
Avian β-defensin 6	*AvBD6*	AAAATCTTGCTGTGTGAGGAAC	GGAGTCCACGCAGGTGACATC	55	[322]
Tight Junction Protein 1	*TJP1*	AGCCCCTTGGTAATGTGTGG	CATTTGGTAGTTGCAGGCAG	55	This Study
Claudin 3	*CLD3*	GGGATTTCTACAACCCGCTG	CCAGGTTTTGGGGTCACAGT	56	This Study

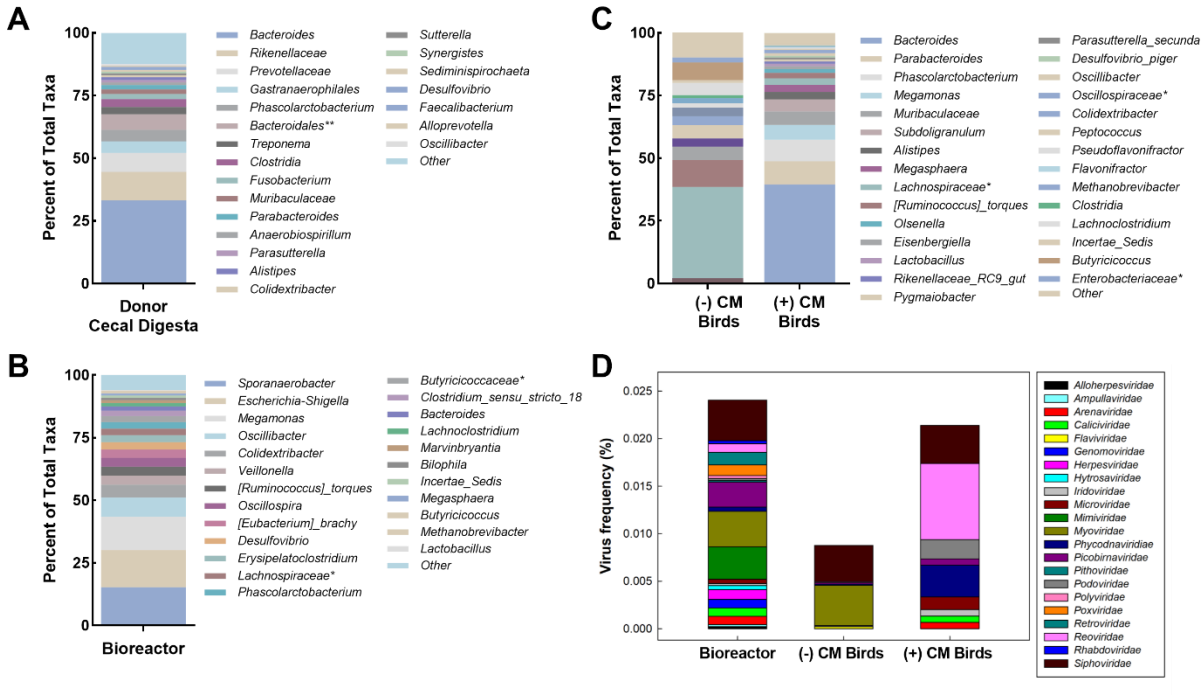

Figure 6.1 Taxonomic classification of bacteria and viruses within bioreactors. (A) Bacterial composition of cecal digesta obtained from adult broiler breeder donors used to population the bioreactors. (B) Composition of bacteria in bioreactors after 12 days. (C) Composition of bacteria in cecal of broilers ± complex microbiota (CM) and not administered *C. perfringens* or corticosterone. (D) Frequencies of viruses in the in the bioreactor and feces obtained from birds ± CM. Viral sequences represented < 0.025% of sequence reads. Bacteria are classified at the genus level unless indicated with asterisk; * represents family level; ** represents order level.

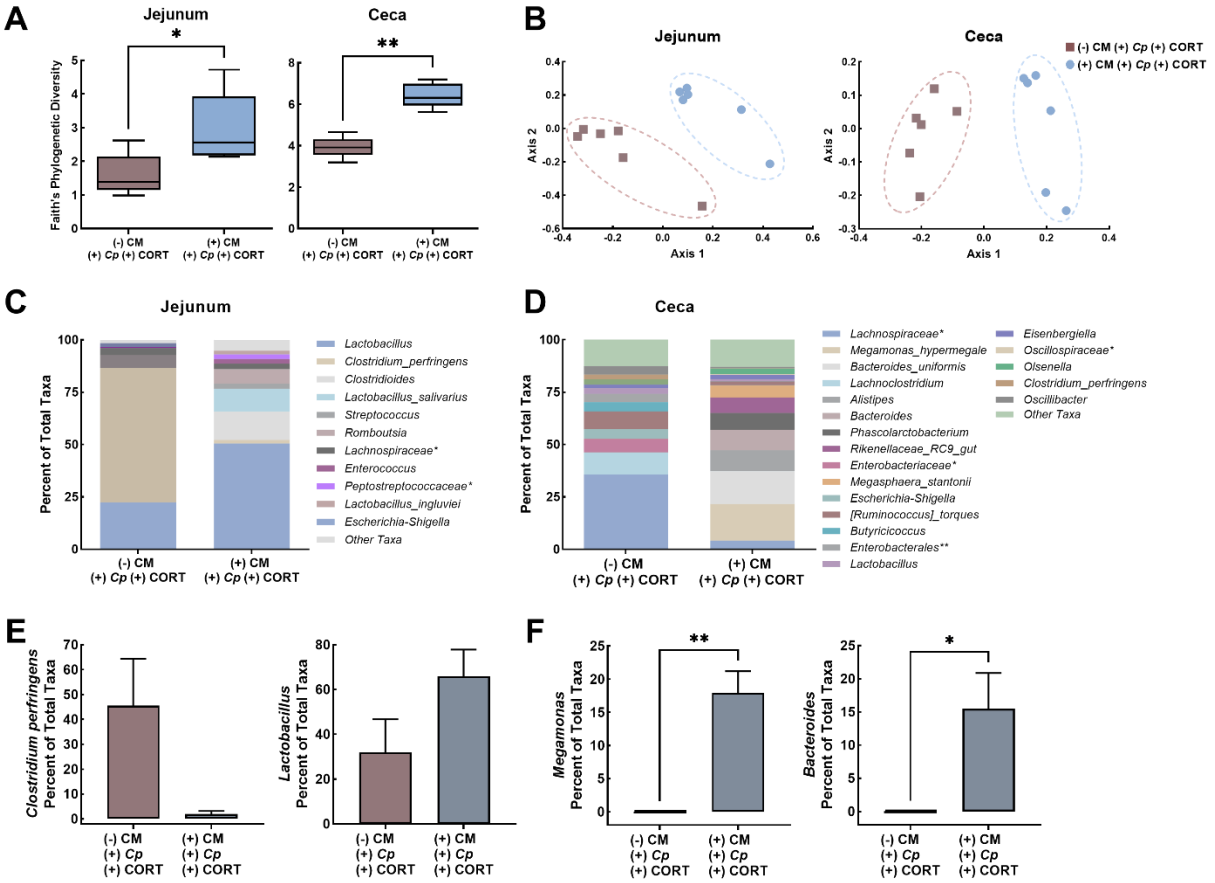

Figure 6.2 Bacterial community structure in jejunal and cecal digesta obtained from chickens. (A) Faith's phylogenetic diversity; * $P < 0.050$ and ** $P < 0.001$. (B) Unweighted UniFrac PCoA, in which differences in β-diversity were observed in both the jejunum ($P = 0.004$) and ceca ($P = 0.006$). (C) Percent relative abundance of bacterial taxa in jejunal digesta (taxa representing >1% of total shown). (D) Percent relative abundance of bacterial taxa in cecal digesta (taxa representing >3% of total shown). Taxa represented at genus level in C-D unless indicated; asterisks in C-D represent * family level and ** order level. (E) Percent abundance of *Clostridium perfringens* and *Lactobacillus* in the jejunal digesta; no differences were observed ($P \geq 0.068$). (F) Percent abundance of *Megamonas* and *Bacteroides* in the cecal digesta. * $P < 0.050$ and ** $P < 0.001$.

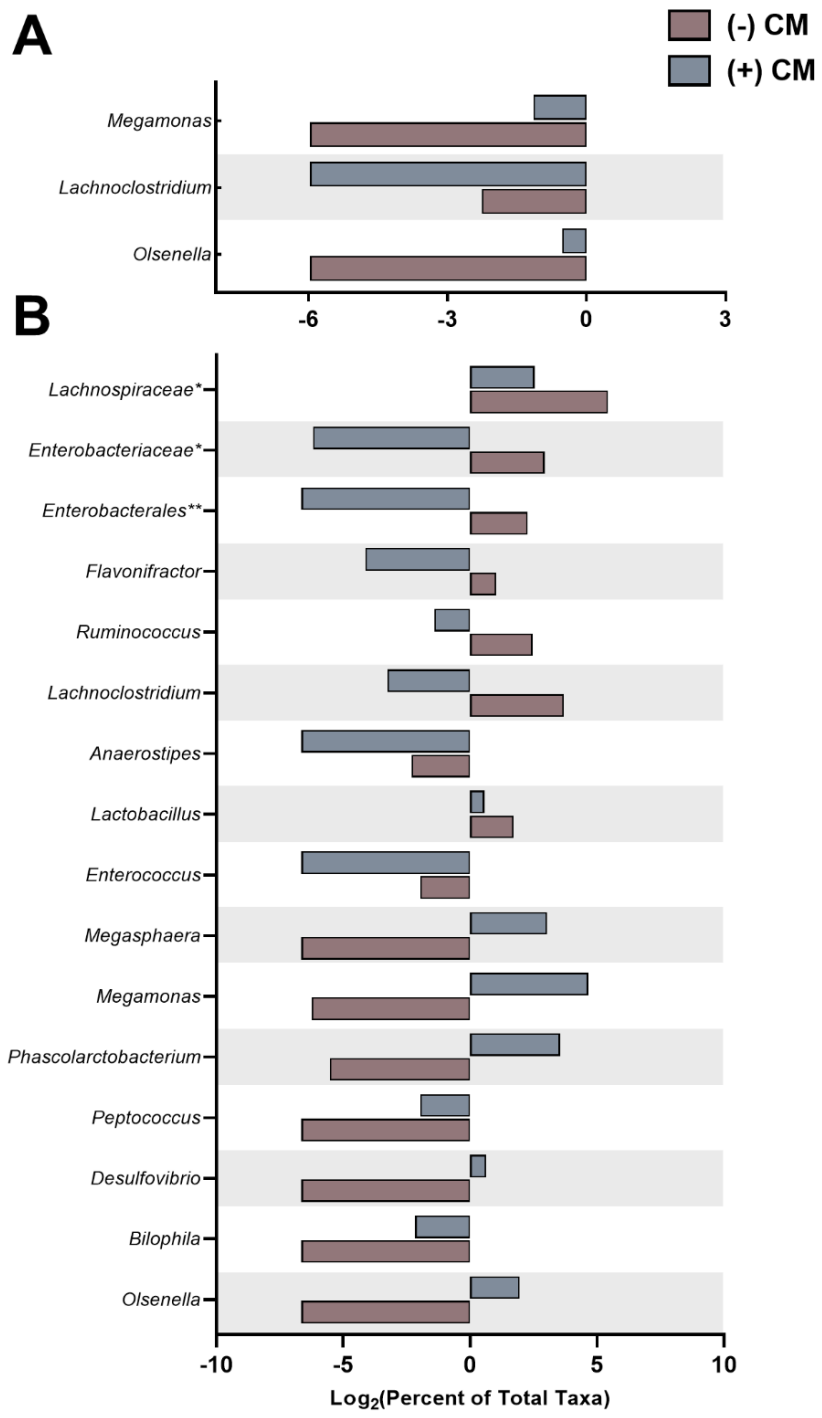

Figure 6.3 Differentially abundant bacterial taxa in jejunal and cecal digesta from broilers as determined by ALDEx2 analysis. Plots represent changes in abundance among birds ± complex microbiota (CM) (i.e. in birds inoculated with *Clostridium perfringens* and administered corticosterone). (A) Jejunum. (B) Ceca. Taxa are presented at a genus level of resolution unless indicated otherwise (* Family and ** Order).

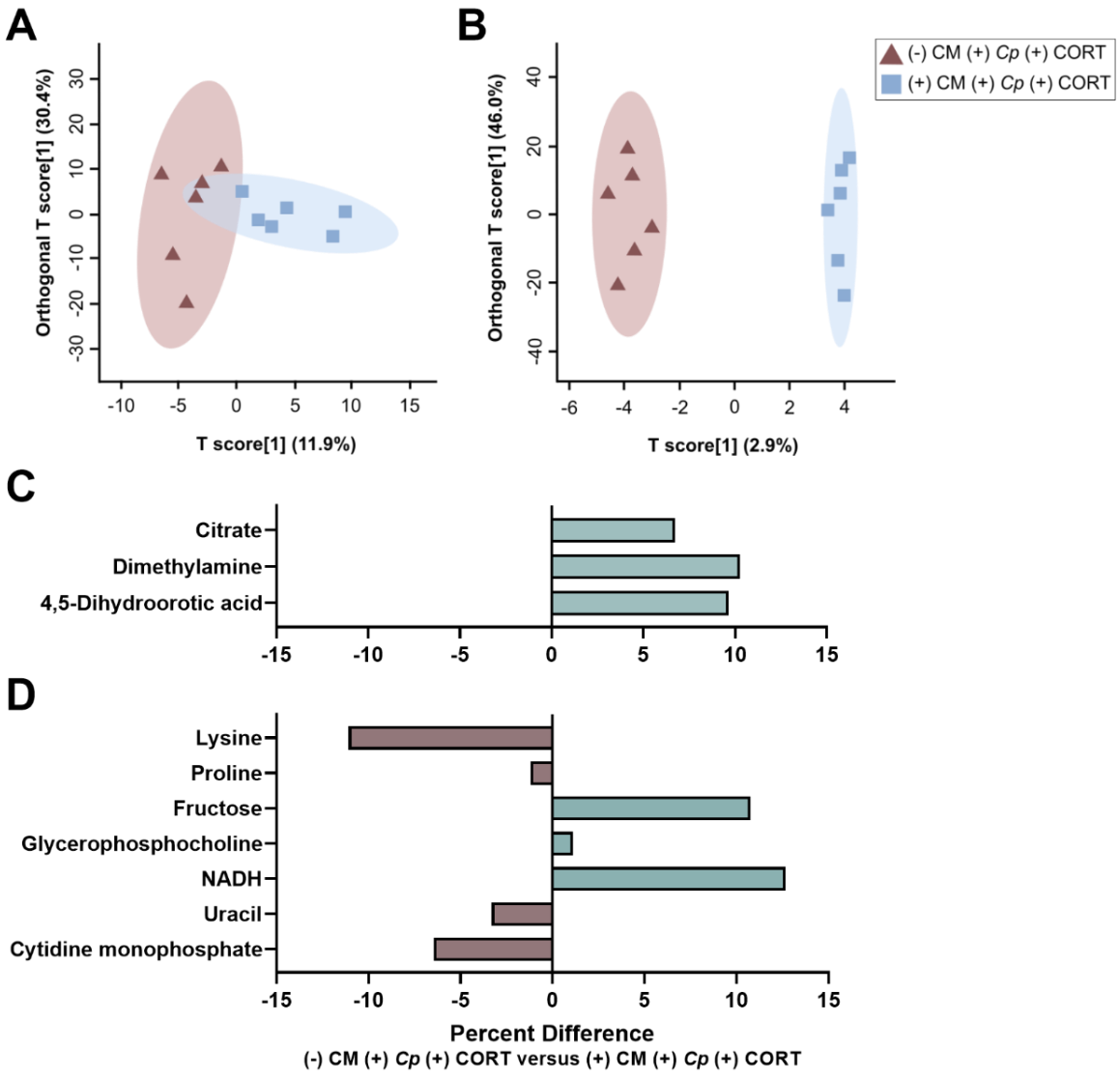

Figure 6.4 Metabolite profiles of jejunal digesta and jejunal tissue from broilers. Plots represent changes in the metabolome among birds ± complex microbiota (CM) (i.e. in birds inoculated with *Clostridium perfringens* [*Cp*] and administered corticosterone [CORT]). (A) Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) of the metabolites in jejunal digesta. (B) OPLS-DA of metabolites in the in jejunal tissue. For A and B, each triangle or square represents one bird, and data were plotted using metabolites identified to be significant by Mann-Whitney U test and/or Variable Importance Analysis based on Variable Combination (VIAVC) machine learning. (C) Metabolites that were differential expressed in jejunal digesta of birds ± CM. (D) Metabolites that were differential expressed in jejunal tissue of birds ± CM. Green bars indicate a positive abundance and brown bars indicates negative abundance in birds not treated with CM in comparison to birds that were.

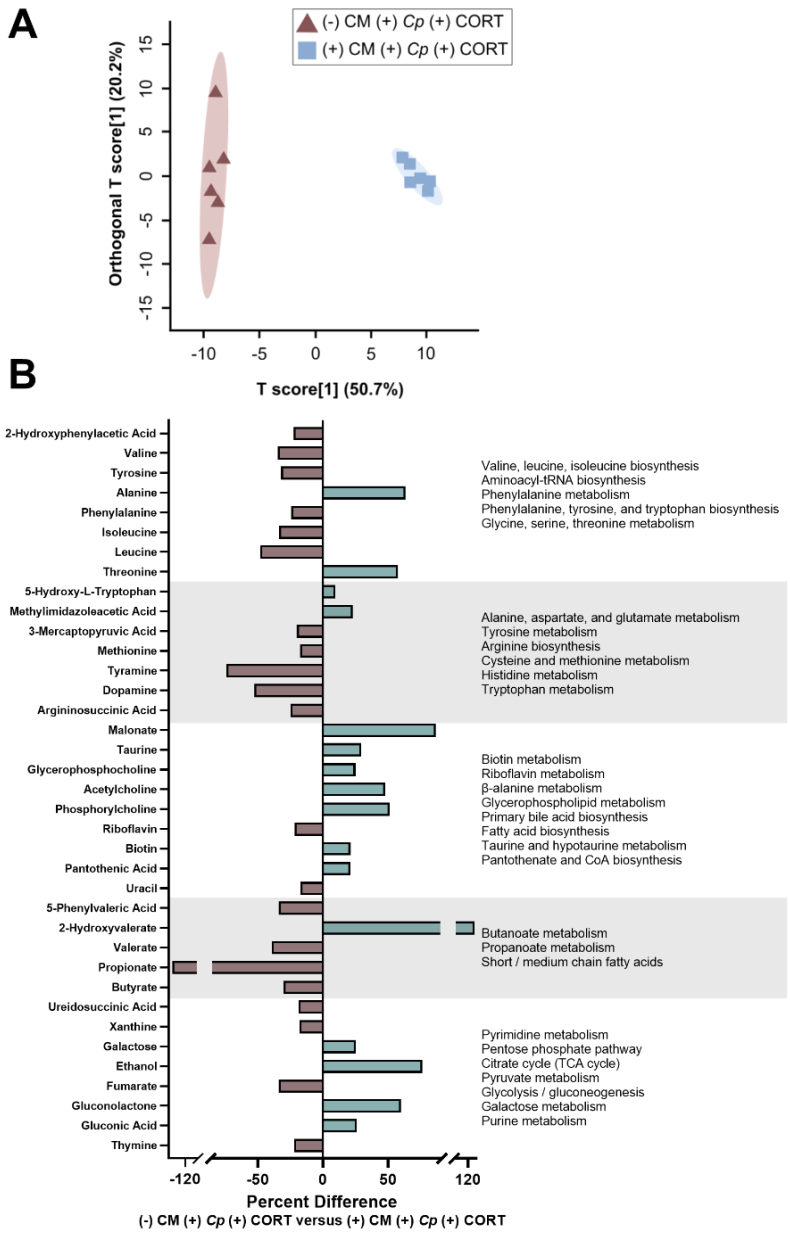

Figure 6.5 Metabolite profiles of cecal digesta from broilers. Plots represent changes in the metabolome among birds ± complex microbiota (CM) (i.e. in birds inoculated with *Clostridium perfringens* [*Cp*] and administered corticosterone [CORT]). (A) Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) of metabolites, in which each triangle or square represents one bird. Metabolites that were differential expressed in jejunal tissue of birds ± CM. Green bars indicate a positive abundance and brown bars indicates negative abundance in birds not treated with complex microbiota in comparison to birds that were. MetaboAnalyst 5.0 was used to associate functional pathways to altered metabolites.

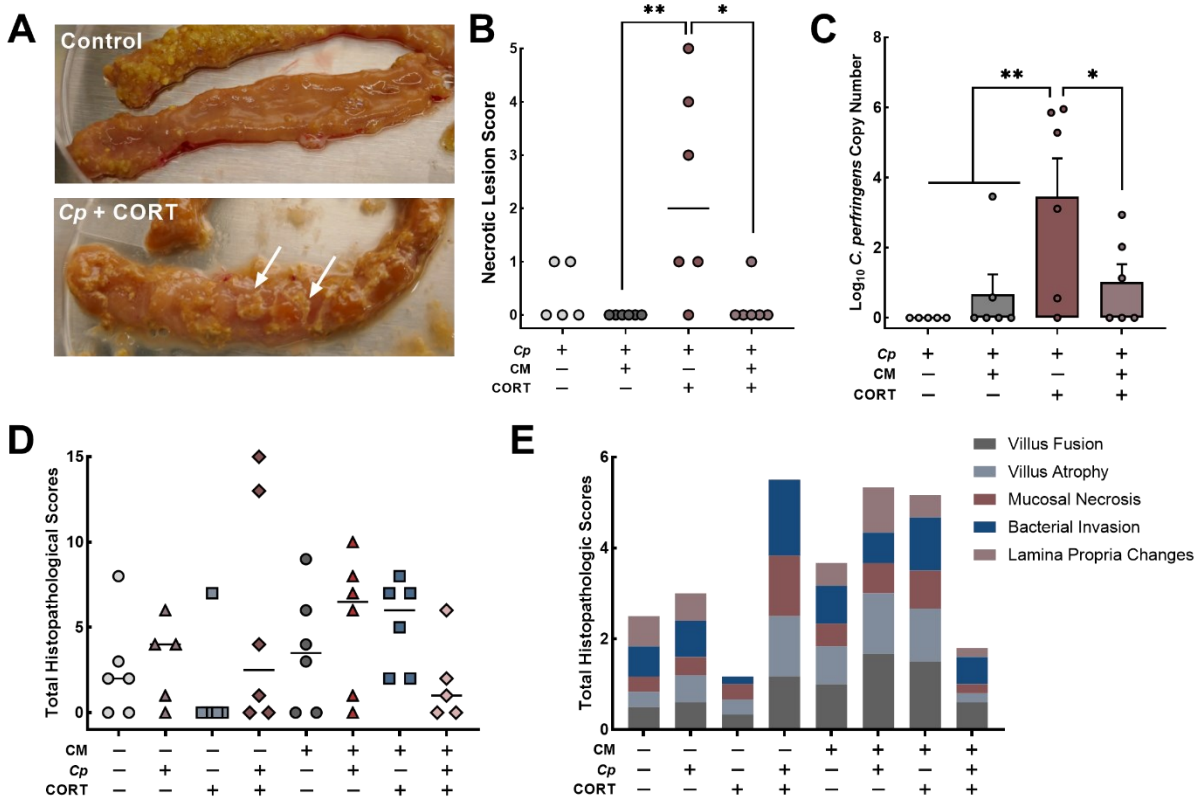

Figure 6.6 Gross pathologies, histopathology, and quantitative PCR for *Clostridium perfringens* in the jejunum. (A) Representative jejunums of control treatment broilers and birds inoculated with *C. perfringens* (*Cp*) and administered corticosterone (CORT). Arrows show fibrin. (B) Jejunal necrotic lesion scores. (C) Densities of *C. perfringens* in jejunal digesta. (D) Total histopathological scores. (E) Stacked bar plot showing average scores of the various histopathological parameters examined. *P ≤ 0.050. **P ≤ 0.010.

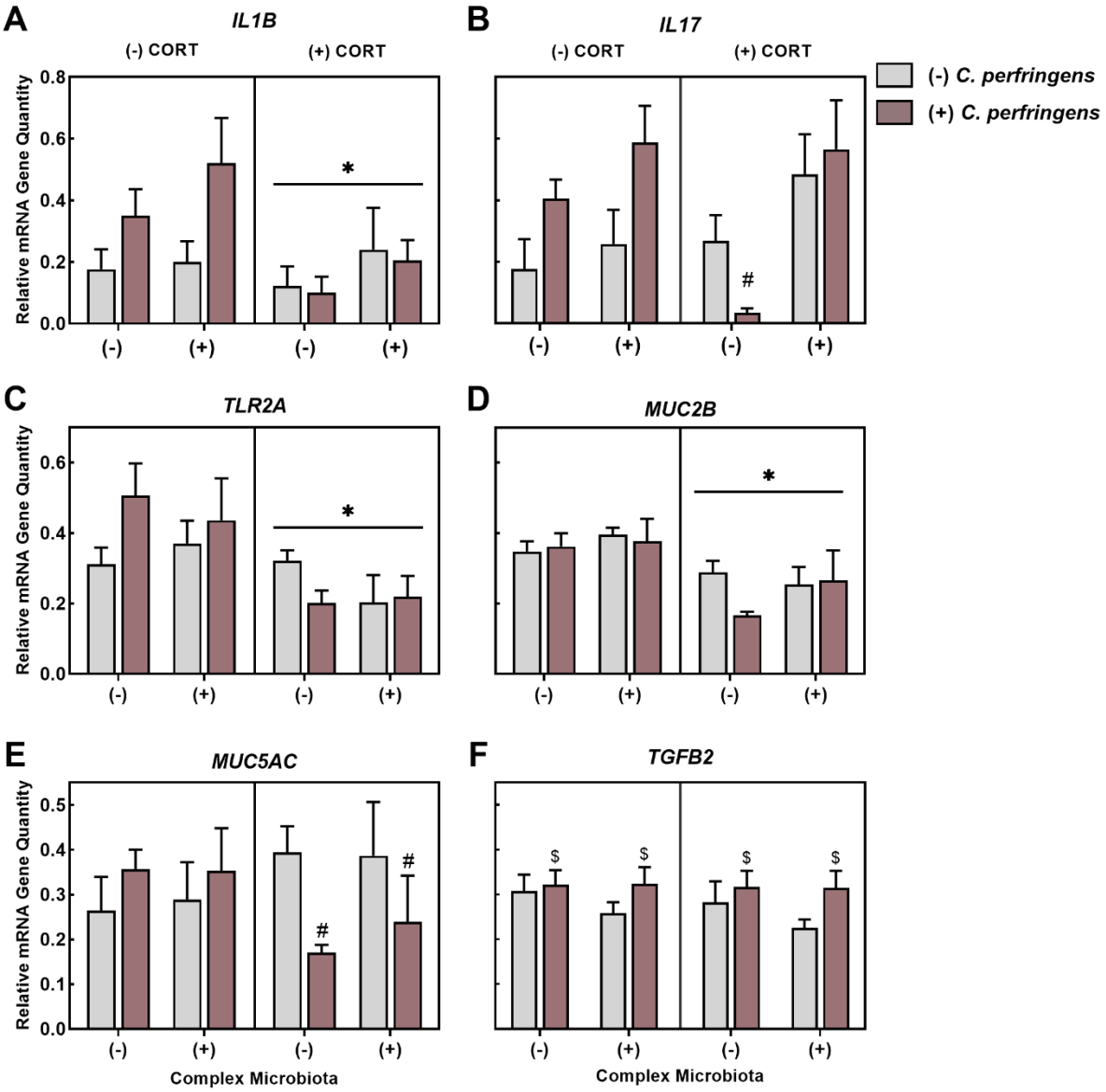

Figure 6.7 Impacts of corticosterone (CORT), *Clostridium perfringens*, and complex microbiota on jejunal epithelium responses. Relative mRNA gene quantities in the jejunum of *IL1B*, *IL17*, *TLR2A*, *MUC2B*, *MUC5AC, and TGFB2* at 2 days post-inoculation with *C. perfringens*. *P ≤ 0.050 for the CORT treatment; $^{\#}$P ≤ 0.040 for a *C. perfringens* x CORT interaction; and $^{\$}$P = 0.045 for *C. perfringens* challenge.

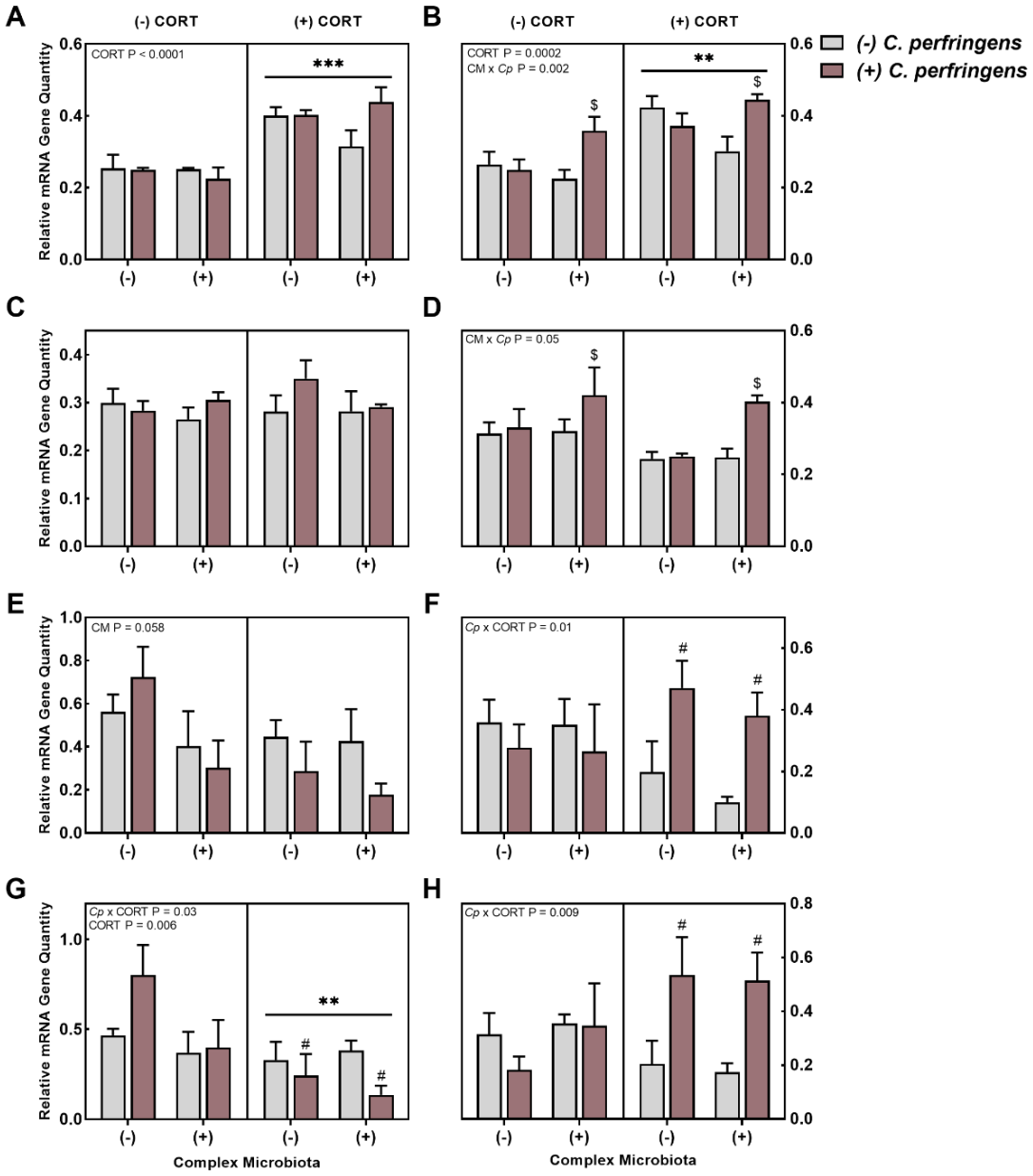

Figure 6.8 Impacts of corticosterone (CORT), *Clostridium perfringens* (*Cp*), and complex microbiota (CM) on relative quantities of genes encoding to tight junction proteins and antimicrobial peptides in the jejunum of broilers. Data from two- and four-days post-inoculation (p.i.) with *C. perfringens* show on left and right sides, respectively. (A) *CLD3* mRNA quantities at 2 days p.i. with *C. perfringens*. (B) *CLD3* mRNA quantities at 4 days p.i. (C) *TJP1* mRNA quantities at 2 days p.i. (D) *TJP1* mRNA quantities at 4 days p.i. (E) *AvBD6* mRNA quantities at 2 days p.i. (F) *AvBD6* mRNA quantities at 4 days p.i. (G) *CATH1* mRNA quantities at 2 days p.i. (H) *CATH1* mRNA quantities at 2 days p.i.**P ≤ 0.010 and *** P ≤ 0.001 for the CORT treatment; $^{\#}$P ≤ 0.030 for a *Cp* x CORT interaction; and $^{\$}$P ≤ 0.050 for *Cp* x CM interaction.

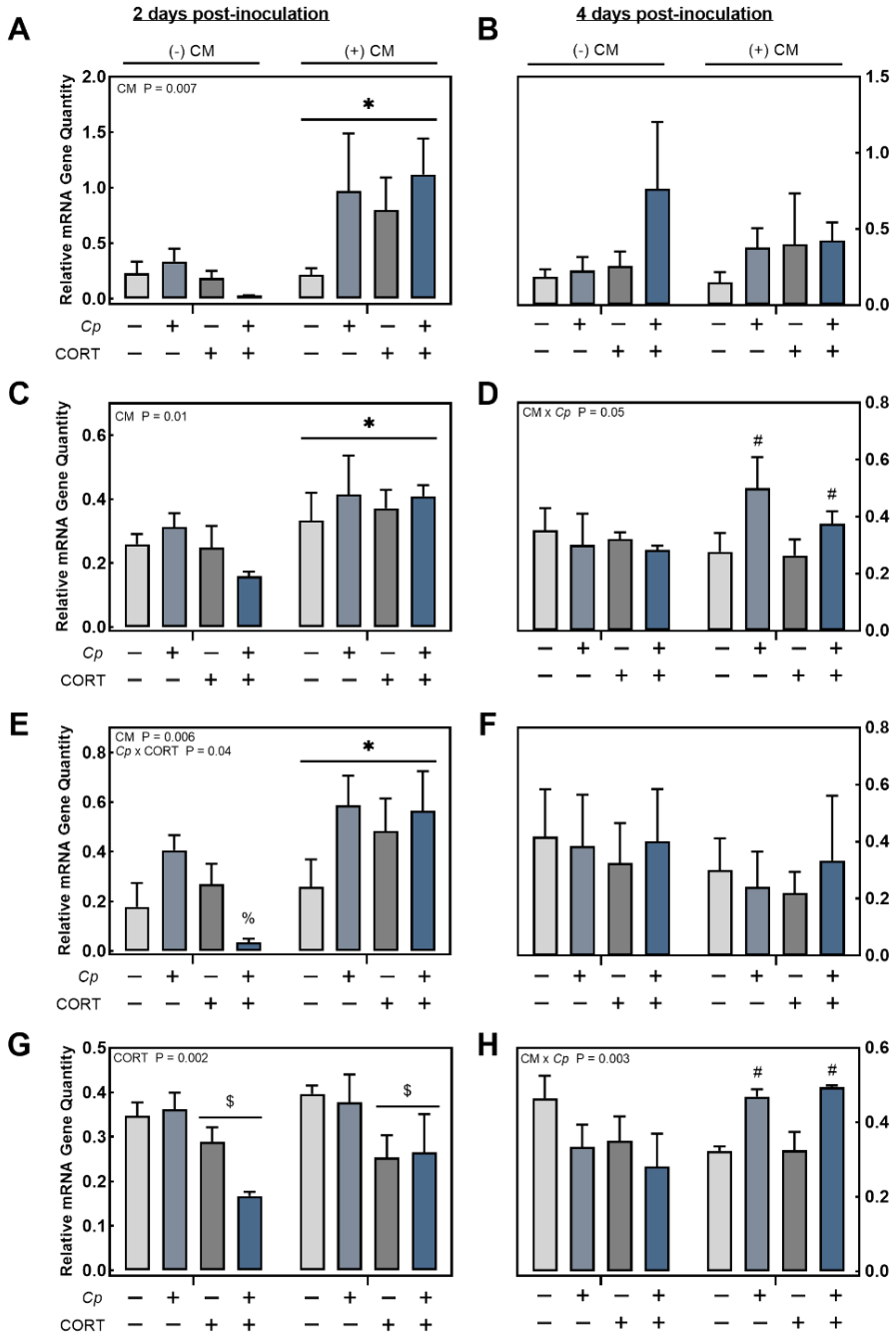

Figure 6.9 Impact of complex microbiota treatment on host responses in the jejunum. Relative mRNA quantities in the jejunum. Data from two- and four-days post-inoculation (p.i.) with *C. perfringens* (*Cp*) show on left and right sides, respectively. (A) *IL22* at 2 days p.i. (B) *IL22* at 4 days p.i. (C) *IL2* at 2 days p.i. (D) *IL2* at 4 days p.i. (E) *IL17* at 2 days p.i. (F) *IL17* at 4 days p.i. (G) *MUC2B* at 2 days p.i. (H) *MUC2B* at 4 days p.i. *P ≤ 0.010 for the complex microbiota (CM) treatment; $^{\$}$P = 0.002 for the corticosterone (CORT) treatment; $^{\%}$P = 0.040 for a *Cp* x CORT interaction; and $^{\#}$P ≤ 0.050 for *Cp* x CM interaction.

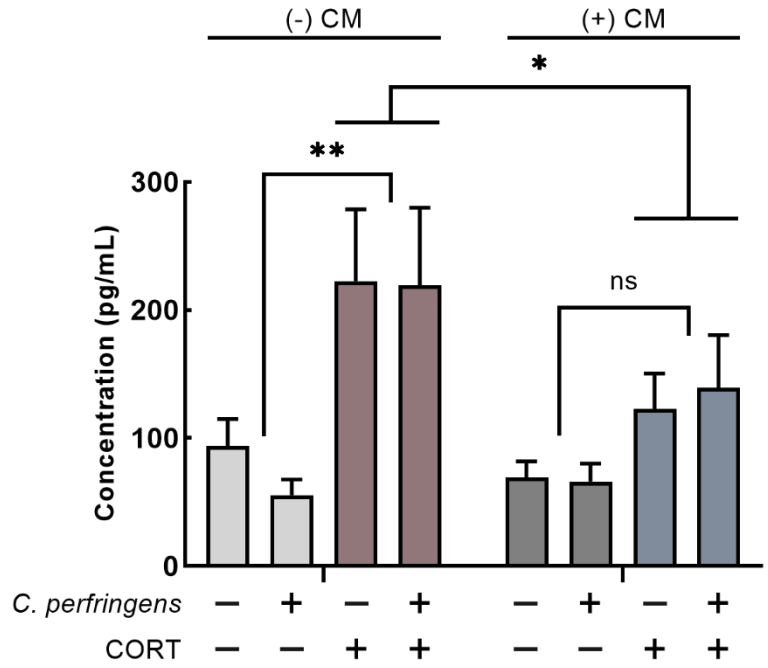

Figure 6.10 Complex microbiota (CM) decreases serum corticosterone (CORT). An ELISA assay was used to quantify CORT in serum. * P = 0.050; ** P = 0.021; ns = not significant.

Chapter 7: General conclusions, knowledge gaps, and future directions

7.1 General conclusions

The prevalence of NE has increased with the reduction of antibiotic use in poultry, and this has initiated the demand for antimicrobial alternatives. A major objective of the research presented in this thesis was to develop an antimicrobial alternative strategy through manipulating microbiota development in young birds. To achieve the outcomes of this objective, a model of NE was needed. Given the importance of managing stress in a post-antibiotic era, the examination of stress as a predisposing factor to NE was evaluated. There are large variations in birds that develop NE and in experimental models can present as inconsistent disease onset that is difficult to standardize and reproduce. Furthermore, other models of NE utilize the use of external co-incitants, such as high protein diet, *Eimeria* spp., or immunosuppressive viruses, with few studies examining intrinsic factors that may be involved in the onset of disease. Thus, I hypothesized that physiological stress experienced by birds can modify host functions in a manner that can predispose them to NE. The stimulation of a stress response can be variable in chickens when exposed to production stressors. Therefore, all studies of this thesis used the stress hormone, corticosterone, as a method of inciting a prescribed physiological stress response. Throughout the progression of this thesis research, modifications to the CORT administration model were made and are outlined in Appendix D (Table S4.1; i.e. changes to dose, layer versus broiler, and method of administration). Importantly, a salient goal of using the corticosterone stress induction model is to elucidate mechanisms and identify cardinal biomarkers, which can then be validated in more variable stress situations (e.g. production settings). The model to be developed also has implications for developing effective non-antibiotic mitigations for NE, and possibly other diseases.

Research presented in chapters 2, 3, and 4 utilized a white leghorn chicken model, and CORT was administered to birds in drinking water. The administration of CORT in water required it to first be dissolved in ethanol. To eliminate any confounding effects of ethanol, my subsequent studies incorporated CORT in-feed (chapters 5 and 6). Moreover, a broiler chicken breed (i.e. Ross 308) was in used in the two latter chapters. In chapters 2 and 3, CORT was administered without *C. perfringens* challenge and at two doses (10 mg/L and 30 mg/L) to determine physiologic and intestinal microbiota alterations during stress. Histopathologic and mRNA gene expression data demonstrated that CORT administration increased hepatic lipid deposition and altered the acute phase response in the liver. In addition, the bursa of Fabricius decreased in size in birds administered CORT, and histopathologic examination showed lymphocyte depletion which, is consistent with the CORT treatment mediating immunosuppression [4]. Metabolite profiles of the kidney, liver, and breast muscle were altered by

CORT treatment at both doses examined. It is noteworthy that the samples obtained from this study were subsequently used to conduct a detailed metabolomics examination to ascertain the impacts of ethanol and CORT on the host. This study was completed in collaboration with M.Sc. student, Catherine (Kate) Brown (University of Lethbridge). The detailed metabolomics examination revealed that birds treated with ethanol alone exhibited metabolite changes suggesting that branched chain amino acid metabolism was altered in the kidney and liver, while amino acid and nitrogen metabolism was altered in breast muscle [323]. These findings emphasized the advantage of administering CORT in feed to eliminate exposure to ethanol (i.e. chapters 5 and 6). Examination of the metabolome showed that sugar and amino acid metabolism was modulated by CORT in all of the tissues examined, which suggests that CORT administration increased glycosylation and gluconeogenic processes. Metabolites (dimethyl glycine, galactose, and carnosine) indicative of reduced meat quality were also shown to increase in birds administered CORT [323].

The research presented in chapter 3 investigated how CORT can influence the microbiota of the small intestine and ceca of white leghorn chickens. Alpha-diversity was decreased in the small intestine of birds administered CORT (30 mg/L) and β-diversity showed the microbial composition of the small intestine and ceca were both modified by CORT administration. Importantly, this study showed CORT administration could increase densities of *C. perfringens* in the small intestine and ceca. Collectively, the results of chapter 2 and 3 demonstrate physiological stress mediated through CORT administration could alter bird metabolism, immune function, and the intestinal microbiota. Importantly, the observation of increased densities of *C. perfringens* in birds administered CORT supports the hypothesis that stress can be a predisposing factor to NE and furthered its investigation in chapters 4 and 5.

A primary objective of the research presented in chapters 4 was to determine to what degree stress promotes the onset of NE by co-challenging white leghorn chickens with CORT and *C. perfringens*, and to shed light on the underlying mechanisms. The CORT treatment increased densities of *C. perfringens* in the small intestinal digesta and mucus. Although no gross necrotic lesions were observed in the white leghorn birds, histopathologic examination of the small intestine confirmed that *C. perfringens* incited pathologic changes to the intestine indicative of subclinical NE. Moreover, *C. perfringens*, and to a lesser extent stress, affected quantifies of mucus in the small intestine, and also altered the composition of mucus glycans. Importantly, growth of the chickens was only adversely affected in birds inoculated with *C. perfringens* that were subject to physiological stress, implicating physiological stress as a key mediator of chronic NE, which can have significant implications on the production health of chickens. This research also implicated mucus utilization by *C. perfringens* in pathogenesis.

Chapter 4 research was followed up with a study that utilized broiler chickens, with the goal of elucidating whether acute NE could be achieved following incitement of a stress response. The results of this study are presented in chapter 5. It was observed that by co-challenging broiler birds with CORT and *C. perfringens* resulted in the manifestation of necrotic lesions in the small intestine indicative of clinical NE. Why leghorn chickens were more resistant to NE was considered. Firstly, host factors involved in response to infection could differ between layers and broilers. For example, it has been demonstrated that β-defensin gene expression differs between broiler breeds (Cobb vs Ross) when challenged with *C. perfringens* [312]. Although this observation of differential β-defensin expression was not between broilers and layers, it suggests that genetic factors involved in immune responses may differ among chicken breeds. Furthermore, broiler and layer birds are bred for different purposes, and other factors, such as metabolic functions, may also factor into disease responses. A major difference in the layers and broilers used in the research presented in this thesis was pre-colonization of the white leghorn chickens with a non-virulent strain of *C. perfringens* (e.g. NetB negative). The white leghorn and Ross 308 eggs that were used in the research presented herein were obtained from different sources. The white leghorn eggs were obtained from a closed SPF flock in Ottawa (Canadian Food Inspection Agency), whereas the broiler eggs were obtained from a commercial hatchery, but originated from an individual broiler breeder farm in southwestern Alberta. As the hatching equipment used at AAFC was fastidiously sanitized with a sporicidal sanitizer suggested that the eggs obtained from the Ottawa flock were contaminated with *C. perfringens* endospores derived from the mother hen, which resulted in the layer chicks becoming colonized by *C. perfringens* on the surface of or within pores of the egg shell during hatching (i.e. before inoculation with a pathogenic strain of the bacterium). In contrast to leghorn chickens, the broiler birds were never colonized by *C. perfringens* after hatch and before inoculation with the pathogenic strain of the bacterium. Since both non-pathogenic and pathogenic strains of *C. perfringens* likely utilize the same nutrient sources within the intestine (e.g. occupy the same ecological niche), it is possible that pre-colonization of the white leghorn chickens by the non-pathogenic strain hindered the pathogenic strains' ability to colonize the intestine and subsequently incite acute disease. Although the exact mechanisms why clinical NE was not manifested in white leghorn chickens remains enigmatic, the model provided valuable information on how stress impacts the host and the development of disease, albeit subclinical disease.

In chapter 6, the stress-incited model of acute NE in Ross 308 broiler chickens presented in chapter 5 was utilized to test the ability of a complex microbiota (CM) administered to day-old chicks to prevent NE, and to elucidate mechanisms involved. The structure of bacterial communities in the jejunum and

ceca of birds administered CM was substantially altered, including a conspicuous increase in diversity of the microbiota within the small and large intestine. Remarkably, a single administration of CM to day-old broiler chicks prevented NE, and no lesions were observed in birds receiving CM. The introduction of CM imparted multiple impacts on the host, including alterations in microbial metabolites within the ceca. In this regard, birds administered CM exhibited elevated SCFA levels in ceca, which corresponded with an increased abundance of anaerobic bacteria that produce SCFAs. It is noteworthy that the production of SCFA are widely thought to protect a host from disease by modulating immune functions, including the enhancement of the epithelial barrier. Stress was observed to decrease the ability of chickens to respond to disease, which facilitated disease progression in birds administered CORT and *C. perfringens*. For example, *TLR2A* and *MUC2B* mRNA responses were decreased by stress. However, birds administered the CM displayed counteracting responses to stress where immune responses were stimulated to fight infection and promote recovery (e.g. increased *IL2*, *IL22*, and *IL17* mRNA expression). Furthermore, exposing birds to the CM at hatch may have bolstered the development of the gut-associated lymphoid tissue and better equipped birds to resist infection. A one time administration of CM to day-old chicks has many advantages over the administration of individual or combinations of probiotic bacteria. For example, probiotics are often repeatedly administered to be effective, which is not economically feasible in broiler chicken production. Future directions and refinement of CM administration as an antibiotic alternative are discussed in more detail below.

7.2 Knowledge gaps

- ***Does the administration of CORT produce similar levels of circulating CORT as production stressors?*** Limited research has evaluated exogenous CORT administration and production stressors in a single study. In the context of circulating CORT, birds recover from an acute production stressor within 1-2 days. A study subjecting birds to either exogenous CORT or production stressors in a single study is warranted to examine CORT responses and determine the comparability of models. Considerations on dose and duration of exposure should be considered.
- ***Do production stressors act synergistically?*** Few studies have examined the impact of multiple concurrent stressors. This may be relevant to production settings where conditions are variable. Production birds are subjected to a range of experiences throughout the production cycle that may be perceived as a stressful stimulus. A single stressor, as is often applied in research studies, may only induce transient changes that can be easily missed in analysis and misinterpreted as inducing no overt pathologies. Broiler birds may be potentially subjected to numerous stressors in the first few weeks of life including travel from the hatchery, learning to feed and drink, potential thermal

stressors, social stress as stocking density increases, and exposure to ammonia to name a few. Limited studies have attempted to simulate multiple stressors, which in combination may result in unwanted pathologies and/or contribute to increase disease susceptibility.

- ***Differences between layer and broilers?*** As mentioned above, it remains unknown whether the white leghorn birds used were more resistant to NE than broiler birds due to genetic or microbial factors. Examining genetic factors between different strains of birds the exert differing levels of disease resistance may aid in the development of therapeutics or provide information for genetic selection. For example, if genetic factors that differ between bird genotypes are determined to be a component to an immune response, perhaps select immunomodulators could be effectively deployed to treat disease or function as a prophylactic.
- ***Does the composition of the microbiota influence stress resilience?*** Increasing research on the bi-directional influence between the brain the microbiome adds additional complexity when interpreting the results of stress studies. Furthermore, the structure of the enteric microbiota varies considerably in different research and barn settings. Factors, such as low microbial diversity, may allow stress to proliferate certain bacteria. In this regard, research presented in this thesis showed that stress altered the composition and reduced the diversity of bacterial communities. Furthermore, research conducted by others has shown that densities of foodborne pathogens, such as *Salmonella enterica* and *Campylobacter jejuni*, increase in birds subjected to stress [190]. An unexpected observation from the research presented in chapter 6 was that the administration of CM to day-old birds was associated with a decrease in serum CORT. Pursuing the mechanisms behind this observation may provide an opportunity to ascertain how microbial community complexity confers both disease resistance and stress resilience.
- ***Importance of bacteria that produce SCFA?*** The production of SCFA and the microbes responsible for their production are thought to regulate enteric inflammation. Alterations to enteric SCFA in chickens with NE, and the administration of SCFAs to chickens in an attempt to control NE has been the subject of research efforts. The research presented herein demonstrated that birds with NE exhibited decreased levels of SCFA and metabolites associated with SCFA production. Given the potential positive influences that SCFA may have on the modulation of barrier function, immune, and stress responses, research that examines the advantages of including SCFA-producing autochthonous bacterial in probiotic regimes, and elucidation of the mechanisms by which these taxa mediate infection by *C. perfringens* and subsequent disease development, is warranted.

- ***Molecular function of glucocorticoids in poultry?*** Mammalian research has provided insights into the mechanistic functions of glucocorticoids at a molecular level of resolution. It remains to be determined if the same or similar mechanisms of glucocorticoid regulation and function occur in chickens. There are some notable regulatory components absent in poultry (i.e. GLUT4). Additionally, HPA function in birds has been proposed to respond differently than mammals. Understanding the molecular mechanisms in poultry could provide better insights as to how stress responses can be regulated and managed, and such research should be emphasized.
- ***Overcoming technology challenges faced by poultry researchers?*** There are limited technologies available to poultry researchers in comparison to researchers that work on mammals. The use of germ-free, gnotobiotic, and knockout models in mice has greatly facilitated elucidation of mechanisms, which has greatly facilitated innovation achievement, including the advancement of technologies that have enhance human health and well-being. Furthermore, the development of analytical tools for poultry science, such as antibodies for flow cytometry and cell sorting, will be instrumental in providing key immunological data. Undoubtedly, funding challenges faced by poultry researchers represent an on-going challenge, and limit the availability of some research tools. However, the 'omics' tools, such as metagenomics, transcriptomics, proteomics, and metabolomics, and associated bioinformatics software are becoming more affordable and assessable. Combining these approaches moving forward will continue to advance our knowledge of how stress influences bird health and disease.

7.3 Future directions

My thesis research produced two important outcomes that will continue to be utilized and researched in the future. The first outcome is the development of a novel method to induce NE through the administration of CORT. This model of NE is unique from existing models. Importantly, it is logistical, reproducible, and reliably generates subclinical or acute NE in chickens, depending on the chicken breed used. The acute model of NE developed in my thesis research will be the foundation of on-going and future research conducted by myself and my research team members. In this regard, the model will be used develop innovative alternatives to antimicrobials, including the development of inhibitors to mucolytic enzymes produced by *C. perfringens,* and the deployment of immunomodulatory molecules to ameliorate inflammation.

The second salient outcome of my research is the demonstration of the ability of single administration of a complex microbiota containing autochthonous bacteria to day-old broiler chicks to preventing NE later in life. Moreover, my research used a bioreactor to generate a CM in which viral

pathogens were reduced or possibly eliminated. The reliance on environmental acquisition of microorganisms colonizing the intestines of chickens can result in a low diversity microbiota early in life, which may have profound impacts on bird health, including susceptibility to important pathogens. Evidence from my research showed that introducing a microbiota to chicks immediately after hatch can impart a positive health outcome, and this strategy may prove to be an important component to antibiotic alternative regimes. Future research should identify key bacteria within the community that provide benefits to the host. Although disease resistance was the focus in this thesis, identifying other microbes that facilitate nutrient digestibility, immune stimulation, and stress resilience would be ideal candidates for next-generation community probiotics. One strategy to developing future applications is employing a systems-based approach to identifying a model microbial community where both composition and function are considered through 'omics' methods. Furthermore, examining and verifying interactions among microbial taxa should be emphasized. For example, determining the degree to which *Lactobacillus* species provide lactate to butyrate-producing bacteria. The application of next-generation community probiotics faces challenges. Current products that claim to be 'complex probiotics' do exist. These products are often dissolving in water for application, which would presumably reduce the success of obligate anaerobes being colonized. These bacteria that are crucial for colonization resistance. Although the colonization of chicken ceca stabilizes at 3-4 weeks-of-age, my research demonstrated that expediting the establishment of functional microbiota at an earlier age conferred beneficial outcomes for bird health. Although the mechanisms responsible were not fully explored, my research showed that enteric bacteria can promote stress resilience. Collectively, the results of my research demonstrate the influential nature that the intestinal microbiota has on the ability of the host to protect itself from stress and stress-mediated disease. The development of next-generation community probiotics should consider beneficial microbe-microbe and microbe-host interactions in selecting microbes for a standardized composition. Additionally, the development of methods to effectively propagate bacteria free of pathogens, and to logistically deliver bacteria without significant loss of viability with the goal of expediting the establishment of a functional microbiota in chicks and thereby enhance bird health and production performance is needed.

References

1. Wade, B. and A. Keyburn, *The true cost of necrotic enteritis.* World Poult, 2015. **31**(7): p. 16-7.
2. Tsiouris, V.V., *Poultry management: a useful tool for the control of necrotic enteritis in poultry.* Avian Pathol, 2016: p. 1-10.
3. Scanes, C.G., *Biology of stress in poultry with emphasis on glucocorticoids and the heterophil to lymphocyte ratio.* Poult Sci, 2016. **95**(9): p. 2208-15.
4. Shini, S., et al., *Understanding stress-induced immunosuppression: exploration of cytokine and chemokine gene profiles in chicken peripheral leukocytes.* Poult Sci, 2010. **89**(4): p. 841-51.
5. Apajalahti, J. and A. Kettunen, *Avian Gut Function in Health and Disease. Chapter 8: Microbes of the Chicken Gastrointestinal Tract*. Poultry Science Symposium Series, ed. G.C. Perry. Vol. 28. 2006: Carfax Publishing Company.
6. Ballou, A.L., et al., *Development of the chick microbiome: how early exposure influences future microbial diversity.* Front Vet Sci, 2016. **3**: p. 2.
7. Smits, L.P., et al., *Therapeutic potential of fecal microbiota transplantation.* Gastroenterology, 2013. **145**(5): p. 946-953.
8. Oakley, B.B., et al., *The chicken gastrointestinal microbiome.* FEMS Microbiol Lett, 2014. **360**(2): p. 100-12.
9. Niederwerder, M.C., *Fecal microbiota transplantation as a tool to treat and reduce susceptibility to disease in animals.* Vet Immunol Immunopathol, 2018. **206**: p. 65-72.
10. Newton, R., *Molecular mechanisms of glucocorticoid action: what is important?* Thorax, 2000. **55**(7): p. 603-613.
11. Payne, D. and I. Adcock, *Molecular mechanisms of corticosteroid actions.* Paediatr Respir, 2001. **2**(2): p. 145-150.
12. Ray, A. and K.E. Prefontaine, *Physical association and functional antagonism between the p65 subunit of transcription factor NF-kappa B and the glucocorticoid receptor.* Proc Natl Acad Sci, 1994. **91**(2): p. 752-756.
13. Heck, S., et al., *A distinct modulating domain in glucocorticoid receptor monomers in the repression of activity of the transcription factor AP-1.* The EMBO journal, 1994. **13**(17): p. 4087-4095.
14. Ito, K., P.J. Barnes, and I.M. Adcock, *Glucocorticoid receptor recruitment of histone deacetylase 2 inhibits interleukin-1β-induced histone H4 acetylation on lysines 8 and 12.* Mol Cell Biol, 2000. **20**(18): p. 6891-6903.

15. Auphan, N., et al., *Immunosuppression by glucocorticoids: inhibition of NF-κB activity through induction of IκB synthesis.* Science, 1995. **270**(5234): p. 286-290.
16. Roldan, M., A.J. Rose, and S. Herzig, *Glucocorticoid hormones and energy homeostasis.* Horm Mol Biol Clin Investig, 2014. **19**(2): p. 117-128.
17. Schakman, O., et al., *Glucocorticoid-induced skeletal muscle atrophy.* Int J Biochem Cell Biol, 2013. **45**(10): p. 2163-2172.
18. Reichardt, H.M., et al., *DNA binding of the glucocorticoid receptor is not essential for survival.* Cell, 1998. **93**(4): p. 531-541.
19. Oishi, K., et al., *Genome-wide expression analysis reveals 100 adrenal gland-dependent circadian genes in the mouse liver.* DNA Research, 2005. **12**(3): p. 191-202.
20. Vander Kooi, B.T., et al., *The glucose-6-phosphatase catalytic subunit gene promoter contains both positive and negative glucocorticoid response elements.* J Mol Endocrinol, 2005. **19**(12): p. 3001-3022.
21. Nader, N., et al., *Liver x receptors regulate the transcriptional activity of the glucocorticoid receptor: implications for the carbohydrate metabolism.* PLoS One, 2012. **7**(3).
22. Jiang, K., et al., *Corticosterone administration and dietary glucose supplementation enhance fat accumulation in broiler chickens.* Br Poult Sci, 2008. **49**(5): p. 625-631.
23. Li, Y., et al., *Effects of stress simulated by dexamethasone on jejunal glucose transport in broilers.* Poult Sci, 2009. **88**(2): p. 330-7.
24. Yuan, L., et al., *Corticosterone administration and high-energy feed results in enhanced fat accumulation and insulin resistance in broiler chickens.* Br Poult Sci, 2008. **49**(4): p. 487-495.
25. Garriga, C., et al., *Heat stress increases apical glucose transport in the chicken jejunum.* Am J Physiol Regul Integr Comp Physiol, 2006. **290**(1): p. 195-201.
26. Zhang, L., et al., *Transport stress in broilers: I. Blood metabolism, glycolytic potential, and meat quality.* Poult Sci, 2009. **88**(10): p. 2033-41.
27. Dong, H., et al., *Altered development and protein metabolism in skeletal muscles of broiler chickens (Gallus gallus domesticus) by corticosterone.* Comp Biochem Physiol A Mol Integr Physiol, 2007. **147**(1): p. 189-95.
28. Woods, C.P., J.M. Hazlehurst, and J.W. Tomlinson, *Glucocorticoids and non-alcoholic fatty liver disease.* J Steroid Biochem, 2015. **154**: p. 94-103.
29. Hillgartner, F.B., L.M. Salati, and A.G. Goodridge, *Physiological and molecular mechanisms involved in nutritional regulation of fatty acid synthesis.* Physiol Rev, 1995. **75**(1): p. 47-76.

30. Legrand, P., et al., *Stearoyl-CoA desaturase activity in primary culture of chicken hepatocytes. Influence of insulin, glucocorticoid, fatty acids and cordycepin.* Int J Biochem, 1994. **26**(6): p. 777-785.
31. Patel, R., et al., *LXRβ is required for glucocorticoid-induced hyperglycemia and hepatosteatosis in mice.* J Clin Investig, 2011. **121**(1): p. 431-441.
32. Hussain, M.M., J. Shi, and P. Dreizen, *Microsomal triglyceride transfer protein and its role in apoB-lipoprotein assembly.* J Lipid Res, 2003. **44**(1): p. 22-32.
33. Martin-Sanz, P., J. Vance, and D. Brindley, *Stimulation of apolipoprotein secretion in very-low-density and high-density lipoproteins from cultured rat hepatocytes by dexamethasone.* Biochem, 1990. **271**(3): p. 575-583.
34. Gathercole, L.L., et al., *11β-Hydroxysteroid dehydrogenase 1: translational and therapeutic aspects.* Endocr Rev, 2013. **34**(4): p. 525-555.
35. Dowman, J.K., et al., *Loss of 5α-reductase type 1 accelerates the development of hepatic steatosis but protects against hepatocellular carcinoma in male mice.* Endocrinology, 2013. **154**(12): p. 4536-4547.
36. Zaytsoff, S.J., et al., *Corticosterone-mediated physiological stress modulates hepatic lipid metabolism, metabolite profiles, and systemic responses in chickens.* Sci Rep, 2019. **9:19225**.
37. Lu, Q., J. Wen, and H. Zhang, *Effect of chronic heat exposure on fat deposition and meat quality in two genetic types of chicken.* Poult Sci, 2007. **86**(6): p. 1059-1064.
38. Yuan, L., et al., *Corticosterone administration and high-energy feed results in enhanced fat accumulation and insulin resistance in broiler chickens.* Br Poult Sci, 2008. **49**(4): p. 487-495.
39. Lu, Z., et al., *Increased fat synthesis and limited apolipoprotein B cause lipid accumulation in the liver of broiler chickens exposed to chronic heat stress.* Poult Sci, 2019. **98**(9): p. 3695-3704.
40. Nguyen, P., et al., *Liver lipid metabolism.* J Anim Physiol Anim Nutr, 2008. **92**(3): p. 272-83.
41. Cai, Y., et al., *Increased de novo lipogenesis in liver contributes to the augmented fat deposition in dexamethasone exposed broiler chickens (Gallus gallus domesticus).* Comp Biochem Physiol C Toxicol Pharmacol, 2009. **150**(2): p. 164-9.
42. Sa, R., et al., *Atmospheric ammonia alters lipid metabolism-related genes in the livers of broilers (Gallus gallus).* J Anim Physiol Anim Nutr, 2018. **102**(2): p. e941-e947.
43. Hu, X., et al., *Effects of glucocorticoids on lipid metabolism and AMPK in broiler chickens' liver.* Comp Biochem Physiol B, Biochem Mol Biol, 2019. **232**: p. 23-30.

44. Liu, J., et al., *Exogenous administration of chronic corticosterone affects hepatic cholesterol metabolism in broiler chickens showing long or short tonic immobility.* Comp Biochem Phys A, 2016. **191**: p. 53-58.
45. Duan, Y., et al., *Cholesterol deregulation induced by chronic corticosterone (CORT) stress in pectoralis major of broiler chickens.* Comp Biochem Phys A, 2014. **176**: p. 59-64.
46. Shimizu, N., et al., *Crosstalk between glucocorticoid receptor and nutritional sensor mTOR in skeletal muscle.* Cell Metab, 2011. **13**(2): p. 170-182.
47. Hasselgren, P.-O., *Glucocorticoids and muscle catabolism.* Curr Opin Clin Nutr Metab Care, 1999. **2**(3): p. 201-205.
48. Ma, K., et al., *Characterization of 5'-regulatory region of human myostatin gene: regulation by dexamethasone in vitro.* Am J Physiol Endocrinol Metab, 2001. **281**(6): p. E1128-E1136.
49. McFarlane, C., et al., *Myostatin induces cachexia by activating the ubiquitin proteolytic system through an NF-κB-independent, FoxO1-dependent mechanism.* J Cell Physiol, 2006. **209**(2): p. 501-514.
50. Kamei, Y., et al., *Skeletal muscle FOXO1 (FKHR) transgenic mice have less skeletal muscle mass, down-regulated Type I (slow twitch/red muscle) fiber genes, and impaired glycemic control.* J Biol Chem, 2004. **279**(39): p. 41114-41123.
51. JAGOE, R.T., et al., *Skeletal muscle mRNA levels for cathepsin B, but not components of the ubiquitin–proteasome pathway, are increased in patients with lung cancer referred for thoracotomy.* Clinic Sci, 2002. **102**(3): p. 353-361.
52. Sandri, M., et al., *Foxo transcription factors induce the atrophy-related ubiquitin ligase atrogin-1 and cause skeletal muscle atrophy.* Cell, 2004. **117**(3): p. 399-412.
53. Frost, R. and C. Lang, *Regulation of insulin-like growth factor-I in skeletal muscle and muscle cells.* Minerva Endocrinol, 2003. **28**(1): p. 53-73.
54. Jellyman, J.K., et al., *Effects of cortisol and dexamethasone on insulin signalling pathways in skeletal muscle of the ovine fetus during late gestation.* PLoS One, 2012. **7**(12).
55. Wang, H., et al., *Dexamethasone represses signaling through the mammalian target of rapamycin in muscle cells by enhancing expression of REDD1.* J Biol Chem, 2006. **281**(51): p. 39128-39134.
56. Fetterer, R. and P. Allen, *Eimeria tenella infection in chickens: effect on plasma and muscle 3-methylhistidine.* Poult Sci, 2001. **80**(11): p. 1549-1553.

57. Furukawa, K., et al., *Time-course changes in muscle protein degradation in heat-stressed chickens: Possible involvement of corticosterone and mitochondrial reactive oxygen species generation in induction of the ubiquitin–proteasome system.* Gen Comp Endocrinol, 2016. **228**: p. 105-110.
58. Nakashima, K., et al., *Effect of dexamethasone on the expression of atrogin-1/MAFbx in chick skeletal muscle.* Anim Sci J, 2016. **87**(3): p. 405-410.
59. Lv, Z.P., et al., *Glucose and lipid metabolism disorders in the chickens with dexamethasone-induced oxidative stress.* J Anim Physiol Anim Nutr, 2018. **102**(2): p. e706-e717.
60. Laplante, M. and D.M. Sabatini, *mTOR signaling at a glance.* J Cell Sci, 2009. **122**(20): p. 3589-3594.
61. Wang, X., et al., *Glucocorticoids retard skeletal muscle development and myoblast protein synthesis through a mechanistic target of rapamycin (mTOR)-signaling pathway in broilers (Gallus gallus domesticus).* Stress, 2015. **18**(6): p. 686-698.
62. *Avian Immunology*. 2nd Edition ed. Vol. 2nd Edition. 2014: Academic Press.
63. Schat, K.A., B. Kaspers, and P. Kaiser, *Avian Immunology*. 2012: Academic Press.
64. Zhai, M., et al., *Identification of genes related to dexamethasone-induced immunosuppression in chicken thymus using transcriptome analysis.* Vet Sci Res J, 2020. **132**: p. 318-327.
65. Guo, Y., et al., *Identification of genes related to effects of stress on immune function in the spleen in a chicken stress model using transcriptome analysis.* Mol Immunol, 2020. **124**: p. 180-189.
66. Zhang, Y., et al., *Transcriptome profile in bursa of Fabricius reveals potential mode for stress-influenced immune function in chicken stress model.* BMC Genomics, 2018. **19**(1): p. 1-12.
67. Shini, S., et al., *Differential alterations in ultrastructural morphology of chicken heterophils and lymphocytes induced by corticosterone and lipopolysaccharide.* Vet Immunol Immunopathol, 2008. **122**(1-2): p. 83-93.
68. McFarlane, J.M. and S.E. Curtis, *Multiple Concurrent Stressors in Chicks.3. Effects on Plasma Corticosterone and the Heterophil:Lymphocyte Ratio.* Poult Sci, 1989. **68**(4): p. 522-527.
69. Maxwell, M.H., *Avian blood leucocyte responses to stress.* Worlds Poult Sci J, 2007. **49**(01): p. 34-43.
70. Edens, F. and H. Siegel, *Modification of corticosterone and glucose responses by sympatholytic agents in young chickens during acute heat exposure.* Poult Sci, 1976. **55**(5): p. 1704-1712.
71. Cain, D.W. and J.A. Cidlowski, *Immune regulation by glucocorticoids.* Nat Rev Immunol, 2017. **17**(4): p. 233-247.

72. Shini, S. and P. Kaiser, *Effects of stress, mimicked by administration of corticosterone in drinking water, on the expression of chicken cytokine and chemokine genes in lymphocytes.* Stress, 2009. **12**(5): p. 388-99.
73. Poh, T.Y., et al., *Re-evaluation of chicken CXCR1 determines the true gene structure: CXCLi1 (K60) and CXCLi2 (CAF/interleukin-8) are ligands for this receptor.* J Biol Chem, 2008. **283**(24): p. 16408-16415.
74. Shini, S., A. Shini, and P. Kaiser, *Cytokine and chemokine gene expression profiles in heterophils from chickens treated with corticosterone.* Stress, 2010. **13**(3): p. 185-194.
75. Dohms, J.E. and A. Metz, *Stress--mechanisms of immunosuppression.* Vet Immunol Immunopathol, 1991. **30**(1): p. 89-109.
76. Gross, W.B., P.B. Siegel, and R.T. Dubose, *Some effects of feeding corticosterone to chickens.* Poult Sci, 1980. **59**(3): p. 516-522.
77. Hirakawa, R., et al., *Heat stress causes immune abnormalities via massive damage to effect proliferation and differentiation of lymphocytes in broiler chickens.* Front Vet Sci, 2020. **7**: p. 46.
78. Quinteiro-Filho, W., et al., *Heat stress impairs performance parameters, induces intestinal injury, and decreases macrophage activity in broiler chickens.* Poult Sci, 2010. **89**(9): p. 1905-1914.
79. Lyte, M., L. Vulchanova, and D.R. Brown, *Stress at the intestinal surface: catecholamines and mucosa–bacteria interactions.* Cell Tissue Res, 2011. **343**(1): p. 23-32.
80. Noti, M., et al., *TNF suppresses acute intestinal inflammation by inducing local glucocorticoid synthesis.* J Exp Med, 2010. **207**(5): p. 1057-1066.
81. Noti, M., et al., *Lipopolysaccharide induces intestinal glucocorticoid synthesis in a TNFα-dependent manner.* The FASEB journal, 2010. **24**(5): p. 1340-1346.
82. Jarillo-Luna, A., et al., *Effect of restraint stress on the population of intestinal intraepithelial lymphocytes in mice.* Brain Behav Immun, 2008. **22**(2): p. 265-275.
83. Lyte, M. and J.F. Cryan, *Microbial endocrinology: the microbiota-gut-brain axis in health and disease*. Vol. 817. 2014: Springer.
84. Castagliuolo, I., et al., *Acute stress causes mucin release from rat colon: role of corticotropin releasing factor and mast cells.* Am J Physiol Gastrointest Liver Physiol, 1996. **271**(5): p. G884-G892.
85. O'Malley, D., et al., *Distinct alterations in colonic morphology and physiology in two rat models of enhanced stress-induced anxiety and depression-like behaviour.* Stress, 2010. **13**(2): p. 114-122.

86. Söderholm, J.D., et al., *Chronic stress induces mast cell–dependent bacterial adherence and initiates mucosal inflammation in rat intestine.* Gastroenterology, 2002. **123**(4): p. 1099-1108.
87. McGuckin, M.A., et al., *Mucin dynamics and enteric pathogens.* Nat Rev Microbiol, 2011. **9**(4): p. 265-78.
88. Kai, H., et al., *Dexamethasone suppresses mucus production and MUC-2 and MUC-5AC gene expression by NCI-H292 cells.* Am J Physiol Lung Cell Mol Physiol, 1996. **271**(3): p. L484-L488.
89. Barnes, P.J., *Anti-inflammatory actions of glucocorticoids: molecular mechanisms.* Clin Sci, 1998. **94**(6): p. 557-572.
90. Fischer, A., et al., *Glucocorticoids regulate barrier function and claudin expression in intestinal epithelial cells via MKP-1.* Am J Physiol Gastrointest Liver Physiol, 2013. **306**(3): p. G218-G228.
91. Boivin, M.A., et al., *Mechanism of glucocorticoid regulation of the intestinal tight junction barrier.* Am J Physiol Gastrointest Liver Physiol, 2007. **292**(2): p. G590-G598.
92. Quinteiro-Filho, W., et al., *Heat stress decreases expression of the cytokines, avian β-defensins 4 and 6 and Toll-like receptor 2 in broiler chickens infected with Salmonella Enteritidis.* Vet Immunol Immunopathol, 2017. **186**: p. 19-28.
93. Varasteh, S., et al., *Differences in susceptibility to heat stress along the chicken intestine and the protective effects of galacto-oligosaccharides.* PLoS One, 2015. **10**(9): p. e0138975.
94. Hu, X. and Y. Guo, *Corticosterone administration alters small intestinal morphology and function of broiler chickens.* Asian-Australas J Anim Sci, 2008. **21**(12): p. 1773-1778.
95. Lyte, M., *Microbial endocrinology and infectious disease in the 21st century.* Trends Microbiol, 2004. **12**(1): p. 14-20.
96. Stanley, D., R.J. Hughes, and R.J. Moore, *Microbiota of the chicken gastrointestinal tract: influence on health, productivity and disease.* Appl Microbiol Biotechnol, 2014. **98**(10): p. 4301-10.
97. Mignon-Grasteau, S., et al., *Impact of Selection for Digestive Efficiency on Microbiota Composition in the Chicken.* PLoS One, 2015. **10**(8): p. 18.
98. Stanley, D., et al., *Identification of chicken intestinal microbiota correlated with the efficiency of energy extraction from feed.* Vet Microbiol, 2013. **164**(1-2): p. 85-92.
99. Inglis, G.D., et al., *Molecular methods to measure intestinal bacteria: a review.* J AOAC Int, 2012. **95**(1): p. 5-23.
100. Kers, J.G., et al., *Host and environmental factors affecting the intestinal microbiota in chickens.* Front Microbiol, 2018. **9**: p. 235.

101. Zaytsoff, S.J., R.R. Uwiera, and G.D. Inglis, *Physiological Stress Mediated by Corticosterone Administration Alters Intestinal Bacterial Communities and Increases the Relative Abundance of Clostridium perfringens in the Small Intestine of Chickens.* Microorganisms, 2020. **8**(10): p. 1518.
102. Rychlik, I., *Composition and function of chicken gut microbiota.* Animals, 2020. **10**(1): p. 103.
103. Glendinning, L., K.A. Watson, and M. Watson, *Development of the duodenal, ileal, jejunal and caecal microbiota in chickens.* Anim Microbi, 2019. **1**(1): p. 1-11.
104. Danzeisen, J.L., et al., *Modulations of the chicken cecal microbiome and metagenome in response to anticoccidial and growth promoter treatment.* PLoS One, 2011. **6**(11): p. e27949.
105. Stecher, B. and W.D. Hardt, *The role of microbiota in infectious disease.* Trends Microbiol, 2008. **16**(3): p. 107-14.
106. Stecher, B. and W.D. Hardt, *Mechanisms controlling pathogen colonization of the gut.* Curr Opin Microbiol, 2011. **14**(1): p. 82-91.
107. Burkholder, K., et al., *Influence of stressors on normal intestinal microbiota, intestinal morphology, and susceptibility to Salmonella enteritidis colonization in broilers.* Poult Sci, 2008. **87**(9): p. 1734-1741.
108. Wang, X., et al., *Effects of high ambient temperature on the community structure and composition of ileal microbiome of broilers.* Poult Sci, 2018. **97**(6): p. 2153-2158.
109. Shi, D., et al., *Impact of gut microbiota structure in heat-stressed broilers.* Poult Sci, 2019. **98**(6): p. 2405-2413.
110. Zhu, L., et al., *Heat stress mediates changes in fecal microbiome and functional pathways of laying hens.* Appl Microbiol Biotechnol, 2019. **103**(1): p. 461-472.
111. Bailey, M.T., et al., *Exposure to a social stressor alters the structure of the intestinal microbiota: implications for stressor-induced immunomodulation.* Brain Behav Immun, 2011. **25**(3): p. 397-407.
112. Marin, I.A., et al., *Microbiota alteration is associated with the development of stress-induced despair behavior.* Sci Rep, 2017. **7**: p. 43859.
113. Van Immerseel, F., et al., *Clostridium perfringens in poultry: an emerging threat for animal and public health.* Avian Pathol, 2004. **33**(6): p. 537-49.
114. Shojadoost, B., A.R. Vince, and J.F. Prescott, *The successful experimental induction of necrotic enteritis in chickens by Clostridium perfringens: a critical review.* Vet Res, 2012. **43**: p. 74.
115. Lee, K.W., et al., *Avian necrotic enteritis: experimental models, host immunity, pathogenesis, risk factors, and vaccine development.* Poult Sci, 2011. **90**(7): p. 1381-90.

116. Lepp, D., et al., *Identification of novel pathogenicity loci in Clostridium perfringens strains that cause avian necrotic enteritis.* PLoS One, 2010. **5**(5): p. e10795.
117. Greenacre, C.B. and T.Y. Morishita, *Backyard Poultry Medicine and Surgery: A Guide for Veterinary Practitioners*. 2014, Wiley-Blackwell.
118. Williams, R.B., *Intercurrent coccidiosis and necrotic enteritis of chickens: rational, integrated disease management by maintenance of gut integrity.* Avian Pathol, 2005. **34**(3): p. 159-80.
119. Al-Sheikhly, F. and R.B. Truscott, *The pathology of necrotic enteritis of chickens following infusion of crude toxins of Clostridium perfringens into the duodenum.* Avian Dis, 1977. **21**(2): p. 241-55.
120. Al-Sheikhly, F. and R.B. Truscott, *The pathology of necrotic enteritis of chickens following infusion of broth cultures of Clostridium perfringens into the duodenum.* Avian Dis, 1977. **21**(2): p. 230-40.
121. Olkowski, A., et al., *Sub-clinical necrotic enteritis in broiler chickens: novel etiological consideration based on ultra-structural and molecular changes in the intestinal tissue.* Vet Sci, 2008. **85**(3): p. 543-553.
122. Van Immerseel, F., et al., *Rethinking our understanding of the pathogenesis of necrotic enteritis in chickens.* Trends Microbiol, 2009. **17**(1): p. 32-6.
123. Donnenberg, M.S., *Pathogenic strategies of enteric bacteria.* Nature, 2000. **406**(6797): p. 768-74.
124. Collier, C.T., et al., *Effects of tylosin on bacterial mucolysis, Clostridium perfringens colonization, and intestinal barrier function in a chick model of necrotic enteritis.* Antimicrob Agents Chemother, 2003. **47**(10): p. 3311-7.
125. MacMillan, J.L., et al., *Structural analysis of broiler chicken small intestinal mucin O-glycan modification by Clostridium perfringens.* Poult Sci, 2019. **0**: p. 1-15.
126. Prescott, J.F., et al., *The pathogenesis of necrotic enteritis in chickens: What we know and what we need to know. Review.* Avian Pathol, 2016: p. 1-21.
127. Podbielski, A. and B. Kreikemeyer, *Cell density--dependent regulation: basic principles and effects on the virulence of Gram-positive cocci.* Int J Infect Dis, 2004. **8**(2): p. 81-95.
128. Cheung, J.K., et al., *The VirSR two-component signal transduction system regulates NetB toxin production in Clostridium perfringens.* Infect Immun, 2010. **78**(7): p. 3064-72.
129. Yu, Q., et al., *The Agr-like quorum sensing system is required for pathogenesis of necrotic enteritis caused by Clostridium perfringens in poultry.* Infect Immun, 2017. **85**(6).
130. Tsiouris, V., et al., *High stocking density as a predisposing factor for necrotic enteritis in broiler chicks.* Avian Pathol, 2015. **44**(2): p. 59-66.

131. Tsiouris, V., et al., *The effect of cold stress on the pathogenesis of necrotic enteritis in broiler chicks.* Avian Pathol, 2015. **44**(6): p. 430-5.
132. Calefi, A.S., et al., *Heat stress, Eimeria spp. and C. perfringens infections alone or in combination modify gut Th1/Th2 cytokine balance and avian necrotic enteritis pathogenesis.* Vet Immunol Immunopathol, 2019. **210**: p. 28-37.
133. Calefi, A.S., et al., *Effects of long-term heat stress in an experimental model of avian necrotic enteritis.* Poult Sci, 2014. **93**(6): p. 1344-1353.
134. Bojanova, D.P. and S.R. Bordenstein, *Fecal transplants: what is being transferred?* PLoS Biol, 2016. **14**(7): p. e1002503.
135. Donaldson, E.E., et al., *The time-course of broiler intestinal microbiota development after administration of cecal contents to incubating eggs.* PeerJ, 2017. **5**: p. e3587.
136. Siegerstetter, S.-C., et al., *Fecal microbiota transplant from highly feed-efficient donors shows little effect on age-related changes in feed-efficiency-associated fecal microbiota from chickens.* Appl Environ Microbiol, 2018. **84**(2).
137. van der Eijk, J.A., et al., *Early-life microbiota transplantation affects behavioural responses, serotonin and immune characteristics in chicken lines divergently selected on feather pecking.* Sci Rep, 2020. **10**(1): p. 1-13.
138. Richards-Rios, P., et al., *Topical application of adult Cecal contents to eggs transplants spore-forming microbiota but not other members of the microbiota to chicks.* Appl Environ Microbiol, 2020. **86**(5).
139. Nurmi, E. and M. Rantala, *New aspects of Salmonella infection in broiler production.* Nature, 1973. **241**(5386): p. 210-211.
140. Shini, S., A. Shini, and G.R. Huff, *Effects of chronic and repeated corticosterone administration in rearing chickens on physiology, the onset of lay and egg production of hens.* Physiol Behav, 2009. **98**(1-2): p. 73-7.
141. Sandercock, D.A., et al., *Acute heat stress-induced alterations in blood acid-base status and skeletal muscle membrane integrity in broiler chickens at two ages: implications for meat quality.* Poult Sci, 2001. **80**(4): p. 418-425.
142. Leveille, G.A., et al., *Lipid biosynthesis in the chick. A consideration of site of synthesis, influence of diet and possible regulatory mechanisms.* Poult Sci, 1975. **54**(4): p. 1075-1093.
143. Rui, L., *Energy metabolism in the liver.* Comp Physiol, 2011. **4**(1): p. 177-197.
144. Hermier, D., *Lipoprotein metabolism and fattening in poultry.* J Nutr, 1997. **127**(5): p. 805S-808S.

145. Baeza, E., et al., *Pertinent plasma indicators of the ability of chickens to synthesize and store lipids.* J Animal Sci, 2015. **93**(1): p. 107-116.
146. Le Roy, C.I., et al., *NMR-based metabolic characterization of chicken tissues and biofluids: a model for avian research.* Metabolomics, 2016. **12**(10): p. 157.
147. Cockrem, J.F., *Stress, corticosterone responses and avian personalities.* J Ornithol, 2007. **148**: p. S169-S178.
148. Pineiro, M., et al., *Characterisation of the pig acute phase protein response to road transport.* Vet J, 2007. **173**(3): p. 669-74.
149. Qiu, X., et al., *Genetic effects on acute phase protein response to the stresses of weaning and transportation in beef calves.* J Anim Sci, 2007. **85**(10): p. 2367-74.
150. Murata, H., N. Shimada, and M. Yoshioka, *Current research on acute phase proteins in veterinary diagnosis: an overview.* Vet J, 2004. **168**(1): p. 28-40.
151. Chamanza, R., et al., *Acute phase proteins in the domestic fowl.* Worlds Poult Sci J, 1999. **55**(1): p. 61-71.
152. Zulkifli, I., et al., *Acute phase proteins, interleukin 6, and heat shock protein 70 in broiler chickens administered with corticosterone.* Poult Sci, 2014. **93**(12): p. 3112-8.
153. Shini, S., et al., *Biological response of chickens (Gallus gallus domesticus) induced by corticosterone and a bacterial endotoxin.* Comp Biochem Physiol B Biochem Mol Biol, 2008. **149**(2): p. 324-33.
154. Post, J., J.M.J. Rebel, and A. ter Huurne, *Physiological effects of elevated plasma corticosterone concentrations in broiler chickens. An alternative means by which to assess the physiological effects of stress.* Poult Sci, 2003. **82**(8): p. 1313-1318.
155. Brunt, E.M., et al., *Nonalcoholic steatohepatitis: a proposal for grading and staging the histological lesions.* Am J Gastroenterol, 1999. **94**(9): p. 2467.
156. Sharma, J., J. Dohms, and A. Metz, *Comparative pathogenesis of serotype 1 and variant serotype 1 isolates of infectious bursal disease virus and their effect on humoral and cellular immune competence of specific-pathogen-free chickens.* Avian Dis, 1989: p. 112-124.
157. Brown, K., et al., *Antimicrobial growth promoters modulate host responses in mice with a defined intestinal microbiota.* Sci Rep, 2016. **6**: p. 38377.
158. Hellemans, J., et al., *qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data.* Genome Biol, 2007. **8**(2): p. R19.
159. Wu, H., et al., *High-throughput tissue extraction protocol for NMR- and MS-based metabolomics.* Anal Biochem, 2008. **372**(2): p. 204-12.

160. Paxman, E.J., et al., *Prenatal maternal stress from a natural disaster alters urinary metabolomic profiles in Project Ice Storm participants.* Sci Rep, 2018. **8**(1): p. 12932.
161. Veselkov, K.A., et al., *Recursive segment-wise peak alignment of biological 1H NMR spectra for improved metabolic biomarker recovery.* Anal Chem, 2008. **81**(1): p. 56-66.
162. Anderson, P.E., et al., *Dynamic adaptive binning: an improved quantification technique for NMR spectroscopic data.* Metabolomics, 2011. **7**(2): p. 179-190.
163. Chong, J., et al., *MetaboAnalyst 4.0: towards more transparent and integrative metabolomics analysis.* Nucleic acids research, 2018. **46**(W1): p. W486-W494.
164. Goodpaster, A.M., L.E. Romick-Rosendale, and M.A. Kennedy, *Statistical significance analysis of nuclear magnetic resonance-based metabonomics data.* Anal Biochem, 2010. **401**(1): p. 134-143.
165. Yun, Y.-H., et al., *Informative metabolites identification by variable importance analysis based on random variable combination.* Metabolomics, 2015. **11**(6): p. 1539-1551.
166. Wise, E.M. and E.G. Ball, *Malic enzyme and lipogenesis.* Proc Natl Acad Sci, 1964. **52**(5): p. 1255-1263.
167. Horton, J.D., J.L. Goldstein, and M.S. Brown, *SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver.* J Clin Invest, 2002. **109**(9): p. 1125-1131.
168. Siuta-Mangano, P., D. Janero, and M.D. Lane, *Association and assembly of triglyceride and phospholipid with glycosylated and unglycosylated apoproteins of very low density lipoprotein in the intact liver cell.* J Biol Chem, 1982. **257**(19): p. 11463-11467.
169. Mendivil, C.O., et al., *Metabolism of VLDL and LDL containing apolipoprotein C-III and not other small apolipoproteins–R2.* Arterioscler Thromb Vasc Biol, 2010. **30**(2): p. 239.
170. Sundaram, M., et al., *Expression of apolipoprotein C-III in McA-RH7777 cells enhances VLDL assembly and secretion under lipid-rich conditions.* J Lipid Res, 2010. **51**(1): p. 150-161.
171. Griffin, H., G. Grant, and M. Perry, *Hydrolysis of plasma triacylglycerol-rich lipoproteins from immature and laying hens (Gallus domesticus) by lipoprotein lipase in vitro.* Biochem J, 1982. **206**(3): p. 647-654.
172. Hazard, D., et al., *Functional genomics of the muscle response to restraint and transport in chickens.* J Anim Sci, 2011. **89**(9): p. 2717-30.
173. Abasht, B., et al., *Oxidative stress and metabolic perturbations in wooden breast disorder in chickens.* PLoS One, 2016. **11**(4): p. e0153750.
174. Brancaccio, P., G. Lippi, and N. Maffulli, *Biochemical markers of muscular damage.* Clin Chem Lab Med, 2010. **48**(6): p. 757-767.

175. Zhao, Y.Y., *Metabolomics in chronic kidney disease.* Clin Chim Acta, 2013. **422**: p. 59-69.
176. Sundekilde, U.K., et al., *High resolution magic angle spinning NMR spectroscopy reveals that pectoralis muscle dystrophy in chicken is associated with reduced muscle content of anserine and carnosine.* Food Chem, 2017. **217**: p. 151-154.
177. Yang, P., et al., *The expression of carnosine and its effect on the antioxidant capacity of longissimus dorsi muscle in finishing pigs exposed to constant heat stress.* Asian-Australas J Anim Sci, 2014. **27**(12): p. 1763-72.
178. Tomonaga, S., et al., *Effects of high ambient temperature on plasma metabolomic profiles in chicks.* Anim Sci J, 2018. **89**(2): p. 448-455.
179. Schuller-Levis, G.B. and E. Park, *Taurine and its chloramine: modulators of immunity.* Neurochem Res, 2004. **29**(1): p. 117-126.
180. Liu, Y., et al., *Adiponectin corrects high-fat diet–induced disturbances in muscle metabolomic profile and whole-body glucose homeostasis.* Diabetes, 2013. **62**(3): p. 743-752.
181. Peng, M.L., et al., *Metabolomics reveals the mechanism of (-)-hydroxycitric acid promotion of protein synthesis and inhibition of fatty acid synthesis in broiler chickens.* Animal, 2018. **12**(4): p. 774-783.
182. Yamauchi, M., et al., *Urinary Level of L-Fucose as a Marker of Alcoholic Liver Disease.* Alcohol Clin Exp Res, 1993. **17**(2): p. 268-271.
183. Eklund, K.K., K. Niemi, and P. Kovanen, *Immune functions of serum amyloid A.* Crit Rev Immunol, 2012. **32**(4).
184. Georgieva, T.M., et al., *Effects of Escherichia coli infection and Eimeria tenella invasion on blood concentrations of some positive acute phase proteins (haptoglobin (PIT 54), fibrinogen and ceruloplasmin) in chickens.* Rev Med Vet, 2010. **161**(2): p. 84.
185. Xie, H., et al., *Effects of Salmonella typhimurium lipopolysaccharide on broiler chickens.* Poult Sci, 2000. **79**(1): p. 33-40.
186. Roberts, A.B. and M.B. Sporn, *Physiological actions and clinical applications of transforming growth factor-β (TGF-β).* Growth factors, 1993. **8**(1): p. 1-9.
187. Kohl, K.D., *Diversity and function of the avian gut microbiota.* J Comp Physiol B, 2012. **182**(5): p. 591-602.
188. Buffie, C.G. and E.G. Pamer, *Microbiota-mediated colonization resistance against intestinal pathogens.* Nat Rev Immunol, 2013. **13**(11): p. 790-801.

189. Dinan, T.G. and J.F. Cryan, *Regulation of the stress response by the gut microbiota: implications for psychoneuroendocrinology.* Psychoneuroendocrinology, 2012. **37**(9): p. 1369-78.
190. Humphrey, T., *Are happy chickens safer chickens? Poultry welfare and disease susceptibility.* Br Poult Sci, 2006. **47**(4): p. 379-391.
191. Stanley, D., et al., *Changes in the caecal microflora of chickens following Clostridium perfringens challenge to induce necrotic enteritis.* Vet Microbiol, 2012. **159**(1-2): p. 155-62.
192. Virden, W., et al., *The effect of corticosterone-induced stress on amino acid digestibility in Ross broilers.* Poult Sci, 2007. **86**(2): p. 338-342.
193. Zaytsoff, S.J., et al., *Host responses to Clostridium perfringens challenge in a chicken model of chronic stress.* Gut Pathog, 2020. **12**(1): p. 1-16.
194. Bolyen, E., et al., *Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2.* Nat Biotechnol, 2019. **37**(8): p. 852-857.
195. Quast, C., et al., *The SILVA ribosomal RNA gene database project: improved data processing and web-based tools.* Nucleic Acids Res, 2012. **41**(D1): p. D590-D596.
196. Anderson, M., et al., *PERMANOVA+ for PRIMER. Guide to software and statistical methods.* 2008.
197. Fernandes, A.D., et al., *Unifying the analysis of high-throughput sequencing datasets: characterizing RNA-seq, 16S rRNA gene sequencing and selective growth experiments by compositional data analysis.* Microbiome, 2014. **2**(1): p. 15.
198. Douglas, G.M., et al., *PICRUSt2 for prediction of metagenome functions.* Nat Biotechnol, 2020: p. 1-5.
199. Deplancke, B., et al., *Selective growth of mucolytic bacteria including Clostridium perfringens in a neonatal piglet model of total parenteral nutrition.* Am J Clin Nutr, 2002. **76**(5): p. 1117-25.
200. Ley, R.E., D.A. Peterson, and J.I. Gordon, *Ecological and evolutionary forces shaping microbial diversity in the human intestine.* Cell, 2006. **124**(4): p. 837-848.
201. Levin, I.I., et al., *Stress response, gut microbial diversity and sexual signals correlate with social interactions.* Biol Lett, 2016. **12**(6): p. 20160352.
202. Stothart, M.R., et al., *Stress and the microbiome: linking glucocorticoids to bacterial community dynamics in wild red squirrels.* Biol Lett, 2016. **12**(1): p. 20150875.
203. Bailey, M.T., H. Engler, and J.F. Sheridan, *Stress induces the translocation of cutaneous and gastrointestinal microflora to secondary lymphoid organs of C57BL/6 mice.* J Neuroimmunol, 2006. **171**(1-2): p. 29-37.

204. Hooper, L.V. and J.I. Gordon, *Glycans as legislators of host–microbial interactions: spanning the spectrum from symbiosis to pathogenicity.* Glycobiology, 2001. **11**(2): p. 1R-10R.
205. Low, K.E., et al., *The glycoconjugate-degrading enzymes of Clostridium perfringens: tailored catalysts for breaching the intestinal mucus barrier.* Glycobiology, 2020.
206. Clavijo, V. and M.J.V. Flórez, *The gastrointestinal microbiome and its association with the control of pathogens in broiler chicken production: a review.* Poult Sci, 2018. **97**(3): p. 1006-1021.
207. Probert, C.S., et al., *The effect of weaning on the clonality of αβ T-cell receptor T cells in the intestine of GF and SPF mice.* Dev Comp Immunol, 2007. **31**(6): p. 606-617.
208. Ludvigsen, J., B. Svihus, and K. Rudi, *Rearing room affects the non-dominant chicken cecum microbiota, while diet affects the dominant microbiota.* Front Vet Sci, 2016. **3**: p. 16.
209. Sylte, M., L. Chandra, and T. Looft, *Evaluation of disinfectants and antiseptics to eliminate bacteria from the surface of turkey eggs and hatch gnotobiotic poults.* Poult Sci, 2017. **96**(7): p. 2412-2420.
210. Bjerrum, L., et al., *Microbial community composition of the ileum and cecum of broiler chickens as revealed by molecular and culture-based techniques.* Poult Sci, 2006. **85**(7): p. 1151-1164.
211. Keyburn, A.L., et al., *NetB, a new toxin that is associated with avian necrotic enteritis caused by Clostridium perfringens.* PLoS Pathog, 2008. **4**(2): p. e26.
212. Johansson, A., et al., *Genetic diversity and prevalence of netB in Clostridium perfringens isolated from a broiler flock affected by mild necrotic enteritis.* Vet Microbiol, 2010. **144**(1-2): p. 87-92.
213. Shimizu, T., et al., *Complete genome sequence of Clostridium perfringens, an anaerobic flesh-eater.* Proc Natl Acad Sci, 2002. **99**(2): p. 996-1001.
214. Drew, M., et al., *Effects of dietary protein source and level on intestinal populations of Clostridium perfringens in broiler chickens.* Poult Sci, 2004. **83**(3): p. 414-420.
215. Tsiouris, V., et al., *Heat stress as a predisposing factor for necrotic enteritis in broiler chicks.* Avian Pathol, 2018. **47**(6): p. 616-624.
216. Moore, R.J., *Necrotic enteritis predisposing factors in broiler chickens.* Avian Pathol, 2016: p. 1-22.
217. Collier, C., et al., *Coccidia-induced mucogenesis promotes the onset of necrotic enteritis by supporting Clostridium perfringens growth.* Vet Immunol Immunopathol, 2008. **122**(1-2): p. 104-115.
218. Stanley, D., et al., *Differential responses of cecal microbiota to fishmeal, Eimeria and Clostridium perfringens in a necrotic enteritis challenge model in chickens.* PLoS One, 2014. **9**(8): p. e104739.
219. Hermans, P. and K. Morgan, *Prevalence and associated risk factors of necrotic enteritis on broiler farms in the United Kingdom; a cross-sectional survey.* Avian Pathol, 2007. **36**(1): p. 43-51.

220. Freestone, P.P., et al., *Microbial endocrinology: how stress influences susceptibility to infection.* Trends Microbiol, 2008. **16**(2): p. 55-64.
221. Smirnov, A., D. Sklan, and Z. Uni, *Mucin dynamics in the chick small intestine are altered by starvation.* J Nutr, 2004. **134**(4): p. 736-42.
222. Soderholm, J.D. and M.H. Perdue, *II. Stress and intestinal barrier function.* Am J Physiol Gastrointest Liver Physiol, 2001. **280**(1): p. G7-G13.
223. Smith, F., et al., *Early weaning stress impairs development of mucosal barrier function in the porcine intestine.* Am J Physiol Gastrointest Liver Physiol, 2009. **298**(3): p. G352-G363.
224. Pearce, S., et al., *Heat stress reduces barrier function and alters intestinal metabolism in growing pigs.* J Anim Sci, 2012. **90**(suppl_4): p. 257-259.
225. Gross, W. and P.J.A.d. Siegel, *Long-term exposure of chickens to three levels of social stress.* Avian Dis, 1981: p. 312-325.
226. Edens, F.W., *Influence of atmospheric ammonia on serum corticosterone, estradiol-17 and progesterone in laying hens.* Int J Poult Sci, 2015. **14**(8): p. 427-435.
227. Virden, W., et al., *Evaluation of models using corticosterone and adrenocorticotropin to induce conditions mimicking physiological stress in commercial broilers.* Poult Sci, 2007. **86**(12): p. 2485-2491.
228. Jensen, E.C., *Quantitative analysis of histological staining and fluorescence using ImageJ.* Anat Rec, 2013. **296**(3): p. 378-381.
229. Huang, Y., Y. Mechref, and M.V. Novotny, *Microscale nonreductive release of O-linked glycans for subsequent analysis through MALDI mass spectrometry and capillary electrophoresis.* Anal Chem, 2001. **73**(24): p. 6063-6069.
230. Vicaretti, S.D., et al., *Capillary electrophoresis analysis of bovine milk oligosaccharides permits an assessment of the influence of diet and the discovery of nine abundant sulfated analogues.* J Agr Food Chem, 2018. **66**(32): p. 8574-8583.
231. Danyluk, H.J., L.K. Shum, and W.F. Zandberg, *A rapid procedure for the purification of 8-aminopyrene trisulfonate (APTS)-labeled glycans for capillary electrophoresis (CE)-based enzyme assays*, in *Protein-Carbohydrate Interactions*. 2017, Springer. p. 223-236.
232. Wylie, A.D. and W.F. Zandberg, *Quantitation of sialic acids in infant formulas by liquid chromatography–mass spectrometry: an assessment of different protein sources and discovery of new analogues.* J Agr Food Chem, 2018. **66**(30): p. 8114-8123.

233. Dubois, M., et al., *Colorimetric method for determination of sugars and related substances.* Anal Chem, 1956. **28**(3): p. 350-356.
234. Gholamiandehkordi, A.R., et al., *Quantification of gut lesions in a subclinical necrotic enteritis model.* Avian Pathol, 2007. **36**(5): p. 375-382.
235. Quinteiro-Filho, W., et al., *Heat stress impairs performance and induces intestinal inflammation in broiler chickens infected with Salmonella Enteritidis.* Avian Pathol, 2012. **41**(5): p. 421-427.
236. Kaldhusdal, M., et al., *Necrotic enteritis challenge models with broiler chickens raised on litter: evaluation of preconditions, Clostridium perfringens strains and outcome variables.* FEMS Immunol Med Microbiol, 1999. **24**(3): p. 337-343.
237. Kitessa, S.M., et al., *Mucin gene mRNA levels in broilers challenged with Eimeria and/or Clostridium perfringens.* Avian Dis, 2014. **58**(3): p. 408-414.
238. Du, E., et al., *Effects of thymol and carvacrol supplementation on intestinal integrity and immune responses of broiler chickens challenged with Clostridium perfringens.* J Anim Sci Biotechno, 2016. **7**(1): p. 19.
239. Amerongen, A.N., et al., *Sulfomucins in the human body.* Biol Chem, 1998. **379**(1): p. 1-18.
240. Brockhausen, I., *Sulphotransferases acting on mucin-type oligosaccharides.* Biochem Soc Trans, 2003. **31 (2)**: p. 318-325.
241. Huang, Y.-L., et al., *Sialic acid catabolism drives intestinal inflammation and microbial dysbiosis in mice.* Nat Commun, 2015. **6**: p. 8141.
242. Struwe, W.B., et al., *Identification of O-glycan structures from chicken intestinal mucins provides insight into Campylobactor jejuni pathogenicity.* Mol Cellul Proteomics, 2015. **14**(6): p. 1464-1477.
243. Veshnyakova, A., et al., *On the interaction of Clostridium perfringens enterotoxin with claudins.* Toxins, 2010. **2**(6): p. 1336-1356.
244. Mitchell, L.A., et al., *Differential effects of claudin-3 and claudin-4 on alveolar epithelial barrier function.* Am J Physiol Lung Cell Mol Physiol, 2011. **301**(1): p. L40-L49.
245. Mukiza, C.N., J.D.J.I. Dubreuil, and immunity, *Escherichia coli heat-stable toxin b impairs intestinal epithelial barrier function by altering tight junction proteins.* Infect Immun, 2013. **81**(8): p. 2819-2827.
246. Nusrat, A., et al., *Clostridium difficile toxins disrupt epithelial barrier function by altering membrane microdomain localization of tight junction proteins.* Infect Immun, 2001. **69**(3): p. 1329-1336.

247. Osselaere, A., et al., *Deoxynivalenol impairs hepatic and intestinal gene expression of selected oxidative stress, tight junction and inflammation proteins in broiler chickens, but addition of an adsorbing agent shifts the effects to the distal parts of the small intestine.* PLoS One, 2013. **8**(7): p. e69014.

248. Tsukita, S. and M. Furuse, *Occludin and claudins in tight-junction strands: leading or supporting players?* Trends Cell Biol, 1999. **9**(7): p. 268-273.

249. Cario, E., *Barrier-protective function of intestinal epithelial Toll-like receptor 2.* Mucosal Immunol, 2008. **1**(1s): p. S62-S66.

250. Cario, E., G. Gerken, and D. Podolsky, *Toll-like receptor 2 controls mucosal inflammation by regulating epithelial barrier function.* Gastroenterology, 2007. **132**(4): p. 1359-1374.

251. Nerren, J.R., et al., *Expression of the avian-specific toll-like receptor 15 in chicken heterophils is mediated by gram-negative and gram-positive bacteria, but not TLR agonists.* Vet Immunol Immunopathol, 2010. **136**(1-2): p. 151-156.

252. Higgs, R., et al., *Induction of a novel chicken Toll-like receptor following Salmonella enterica serovar Typhimurium infection.* Infect Immun, 2006. **74**(3): p. 1692-1698.

253. Lancaster, G.I., et al., *The physiological regulation of toll-like receptor expression and function in humans.* J Physiol, 2005. **563**(3): p. 945-955.

254. Cario, E., G. Gerken, and D.K. Podolsky, *Toll-like receptor 2 enhances ZO-1-associated intestinal epithelial barrier integrity via protein kinase C.* Gastroenterology, 2004. **127**(1): p. 224-238.

255. Huang, T., et al., *Temporal effects of high fishmeal diet on gut microbiota and immune response in Clostridium perfringens-challenged chickens.* Front Microbiol, 2018. **9**: p. 2754.

256. Fasina, Y.O. and H.S. Lillehoj, *Characterization of intestinal immune response to Clostridium perfringens infection in broiler chickens.* Poult Sci, 2018. **98**(1): p. 188-198.

257. Park, S.S., et al., *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 Dis, 2008. **52**(1): p. 14-22.

258. John, J.L., *The avian spleen: a neglected organ.* Q Rev Biol, 1994. **69**(3): p. 327-351.

259. Hilton, L.S., et al., *Interleukin-2 directly induces activation and proliferation of chicken T cells in vivo.* J Interferon Cytokine, 2002. **22**(7): p. 755-763.

260. Gibson, M.S., P. Kaiser, and M. Fife, *Identification of chicken granulocyte colony-stimulating factor (G-CSF/CSF3): the previously described myelomonocytic growth factor is actually CSF3.* J Interferon Cytokine, 2009. **29**(6): p. 339-344.

261. Hong, Y.H., et al., *Differential gene expression profiles of β-defensins in the crop, intestine, and spleen using a necrotic enteritis model in 2 commercial broiler chicken lines.* Poult Sci, 2012. **91**(5): p. 1081-1088.
262. Compton, M.M., P.S. Gibbs, and L.R. Johnson, *Glucocorticoid activation of deoxyribonucleic acid degradation in bursal lymphocytes.* Poult Sci, 1990. **69**(8): p. 1292-1298.
263. Colditz, I., *Effects of the immune system on metabolism: implications for production and disease resistance in livestock.* Livest Prod Sci, 2002. **75**(3): p. 257-268.
264. Palliyeguru, M., S. Rose, and A. Mackenzie, *Effect of dietary protein concentrates on the incidence of subclinical necrotic enteritis and growth performance of broiler chickens.* Poult Sci, 2010. **89**(1): p. 34-43.
265. McReynolds, J., et al., *Evaluation of immunosuppressants and dietary mechanisms in an experimental disease model for necrotic enteritis.* Poult Sci, 2004. **83**(12): p. 1948-1952.
266. Calefi, A.S., et al., *The gut-brain axis interactions during heat stress and avian necrotic enteritis.* Poult Sci, 2016. **95**(5): p. 1005-14.
267. To, H., et al., *Experimental induction of necrotic enteritis in chickens by a netB-positive Japanese isolate of Clostridium perfringens.* J Vet Med Sci, 2016: p. 16-0500.
268. Tate, R.F., *Correlation between a discrete and a continuous variable. Point-biserial correlation.* Ann Math Stat, 1954. **25**(3): p. 603-607.
269. Aktan, F., *iNOS-mediated nitric oxide production and its regulation.* Life sciences, 2004. **75**(6): p. 639-653.
270. Lee, Y., et al., *Allium hookeri supplementation improves intestinal immune response against necrotic enteritis in young broiler chickens.* Poult Sci, 2018. **97**(6): p. 1899-1908.
271. Zhang, Z., et al., *Effects of cold stress on nitric oxide in duodenum of chicks.* Poult Sci, 2011. **90**(7): p. 1555-1561.
272. Cao, L., et al., *Reduced lesions in chickens with Clostridium perfringens-induced necrotic enteritis by Lactobacillus fermentum 1.2029.* Poult Sci, 2012. **91**(12): p. 3065-3071.
273. Berndt, A., et al., *Chicken cecum immune response to Salmonella enterica serovars of different levels of invasiveness.* Infect Immun, 2007. **75**(12): p. 5993-6007.
274. Quraishi, M.N., et al., *Immunological mechanisms underpinning faecal microbiota transplantation for the treatment of inflammatory bowel disease.* Clin Exp Immunol, 2020. **199**(1): p. 24-38.
275. Dahiya, J.P., et al., *Potential strategies for controlling necrotic enteritis in broiler chickens in post-antibiotic era*. 2006, Anim Feed Sci. p. 60-88.

276. Khalique, A., et al., *Probiotics mitigating subclinical necrotic enteritis (SNE) as potential alternatives to antibiotics in poultry.* Amb Express, 2020. **10**(1): p. 1-10.
277. Wang, H., et al., *Probiotic enhanced intestinal immunity in broilers against subclinical necrotic enteritis.* Front Immunol, 2017. **8**: p. 1592.
278. Qing, X., et al., *Preventing subclinical necrotic enteritis through Lactobacillus johnsonii BS15 by ameliorating lipid metabolism and intestinal microflora in broiler chickens.* Amb Express, 2017. **7**(1): p. 1-12.
279. La Ragione, R., et al., *In vivo characterization of Lactobacillus johnsonii FI9785 for use as a defined competitive exclusion agent against bacterial pathogens in poultry.* Lett Appl Microbiol, 2004. **38**(3): p. 197-205.
280. Teo, A.Y.-L. and H.-M. Tan, *Inhibition of Clostridium perfringens by a novel strain of Bacillus subtilis isolated from the gastrointestinal tracts of healthy chickens.* Appl Environ Microbiol, 2005. **71**(8): p. 4185-4190.
281. De Maesschalck, C., et al., *Effects of Xylo-Oligosaccharides on Broiler Chicken Performance and Microbiota.* Appl Environ Microbiol, 2015. **81**(17): p. 5880-8.
282. Canada, P.H.A.o., *Canadian biosafety standards and guidelines for facilities handling human and terrestrial animal pathogens, prions, and biological toxins*. 2013: Canadian Museum of Civilization/Musee Canadien Des Civilisations.
283. Low, K.E., et al., *Combinatorial glycomic analyses to direct CAZyme discovery for the tailored degradation of canola meal non-starch dietary polysaccharides.* Microorganisms, 2020. **8**(12): p. 1888.
284. Auchtung, J.M., et al., *MiniBioReactor arrays (MBRAs) as a tool for studying C. difficile physiology in the presence of a complex community*, in *Clostridium difficile*. 2016, Springer. p. 235-258.
285. Robinson, C.D., et al., *Epidemic Clostridium difficile strains demonstrate increased competitive fitness compared to nonepidemic isolates.* Infect Immun, 2014. **82**(7): p. 2815-2825.
286. Kozich, J.J., et al., *Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform.* Appl Environ Microbiol, 2013. **79**(17): p. 5112-5120.
287. Kramná, L. and O. Cinek, *Virome sequencing of stool samples*, in *The Human Virome*. 2018, Springer. p. 59-83.
288. Nantel-Fortier, N., et al., *Treatments of porcine fecal samples affect high-throughput virome sequencing results.* Journal of Virological Methods, 2021. **289**: p. 114045.

289. Inglis, G.D., et al., *Expeller-Pressed Canola (Brassica napus) Meal Modulates the Structure and Function of the Cecal Microbiota, and Alters the Metabolome of the Pancreas, Liver, and Breast Muscle of Broiler Chickens.* Animals, 2021. **11**(2): p. 577.
290. Chong, J. and J. Xia, *MetaboAnalystR: an R package for flexible and reproducible analysis of metabolomics data.* Bioinformatics, 2018. **34**(24): p. 4313-4314.
291. Emami, N.K., et al., *Necrotic enteritis in broiler chickens: the role of tight junctions and mucosal immune responses in alleviating the effect of the disease.* Microorganisms, 2019. **7**(8): p. 231.
292. Hofacre, C., et al., *Using competitive exclusion, mannan-oligosaccharide and other intestinal products to control necrotic enteritis.* J Appl Poult Res, 2003. **12**(1): p. 60-64.
293. Antonissen, G., et al., *Microbial shifts associated with necrotic enteritis.* Avian Pathol, 2016: p. 1-14.
294. Wu, S.B., et al., *Two necrotic enteritis predisposing factors, dietary fishmeal and Eimeria infection, induce large changes in the caecal microbiota of broiler chickens.* Vet Microbiol, 2014. **169**(3-4): p. 188-97.
295. Kubasova, T., et al., *Contact with adult hen affects development of caecal microbiota in newly hatched chicks.* PLoS One, 2019. **14**(3): p. e0212446.
296. Garde, S., et al., *Susceptibility of Clostridium perfringens to antimicrobials produced by lactic acid bacteria: Reuterin and nisin.* Food Control, 2014. **44**: p. 22-25.
297. Harrison, A. and P. Hansen, *Bacteroides hypermegas nov. spec.* Antonie van Leeuwenhoek, 1963. **29**(1): p. 22-28.
298. Sengupta, R., et al., *The role of cell surface architecture of lactobacilli in host-microbe interactions in the gastrointestinal tract.* Mediat Inflamm, 2013. **2013**.
299. Sergeant, M.J., et al., *Extensive microbial and functional diversity within the chicken cecal microbiome.* PLoS One, 2014. **9**(3): p. e91941.
300. Clench, M.H. and J.R. Mathias, *The avian cecum: a review.* The Wilson Bulletin, 1995: p. 93-121.
301. Furness, J.B., et al., *The gut as a sensory organ.* Nat Rev Gastroenterol Hepatol, 2013. **10**(12): p. 729.
302. Maki, J.J. and T. Looft, *Megasphaera stantonii sp. nov., a butyrate-producing bacterium isolated from the cecum of a healthy chicken.* Int J Syst Evol Micr, 2018. **68**(11): p. 3409-3415.
303. Eeckhaut, V., et al., *Anaerostipes butyraticus sp. nov., an anaerobic, butyrate-producing bacterium from Clostridium cluster XIVa isolated from broiler chicken caecal content, and emended description of the genus Anaerostipes.* Int J Syst Evol Micr, 2010. **60**(5): p. 1108-1112.

304. Morrison, D.J. and T. Preston, *Formation of short chain fatty acids by the gut microbiota and their impact on human metabolism.* Gut Microbes, 2016. **7**(3): p. 189-200.
305. Mogensen, T.H., *Pathogen recognition and inflammatory signaling in innate immune defenses.* Clin Microbiol Rev, 2009. **22**(2): p. 240-73, Table of Contents.
306. Veldhoen, M., *Interleukin 17 is a chief orchestrator of immunity.* Nat Immunol, 2017. **18**(6): p. 612.
307. Ogura, H., et al., *Interleukin-17 promotes autoimmunity by triggering a positive-feedback loop via interleukin-6 induction.* Immunity, 2008. **29**(4): p. 628-636.
308. Freitas, M., et al., *Microbial–host interactions specifically control the glycosylation pattern in intestinal mouse mucosa.* Histochem Cell Biol, 2002. **118**(2): p. 149-161.
309. Fukushima, K., et al., *Colonization of microflora in mice: mucosal defense against luminal bacteria.* J Gastroenterol, 1999. **34**(1): p. 54-60.
310. Bommineni, Y.R., et al., *Immune regulatory activities of fowlicidin-1, a cathelicidin host defense peptide.* Mol Immunol, 2014. **59**(1): p. 55-63.
311. Martin-Ezquerra, G., et al., *Psychological stress regulates antimicrobial peptide expression by both glucocorticoid and β-adrenergic mechanisms.* Eur J Dermatol, 2011. **21**(2): p. 48-51.
312. Hong, Y., et al., *Differential gene expression profiles of β-defensins in the crop, intestine, and spleen using a necrotic enteritis model in 2 commercial broiler chicken lines.* Poult Sci, 2012. **91**(5): p. 1081-1088.
313. Latorre, J.D., et al., *Evaluation of the epithelial barrier function and ileal microbiome in an established necrotic enteritis challenge model in broiler chickens.* Front Vet Sci, 2018. **5**: p. 199.
314. Kaiser, P., *Advances in avian immunology--prospects for disease control: a review.* Avian Pathol, 2010. **39**(5): p. 309-24.
315. Bar-Shira, E., D. Sklan, and A. Friedman, *Establishment of immune competence in the avian GALT during the immediate post-hatch period.* Dev Comp Immunol, 2003. **27**(2): p. 147-57.
316. Yadav, V., et al., *Colonic bacterial metabolism of corticosteroids.* Int J Pharm, 2013. **457**(1): p. 268-274.
317. van de Wouw, M., et al., *Short-chain fatty acids: microbial metabolites that alleviate stress-induced brain–gut axis alterations.* J Physiol, 2018. **596**(20): p. 4923-4944.
318. Ait-Belgnaoui, A., et al., *Prevention of gut leakiness by a probiotic treatment leads to attenuated HPA response to an acute psychological stress in rats.* Psychoneuroendocrinology, 2012. **37**(11): p. 1885-1895.

319. Petrof, E.O., et al., *Stool substitute transplant therapy for the eradication of Clostridium difficile infection:'RePOOPulating'the gut.* Microbiome, 2013. **1**(1): p. 1-12.
320. Khoruts, A., M.J. Sadowsky, and M.J. Hamilton, *Development of fecal microbiota transplantation suitable for mainstream medicine.* Clin Gastroenterol Hepatol, 2015. **13**(2): p. 246-250.
321. Jiang, Z., T.J. Applegate, and A.C. Lossie, *Cloning, annotation and developmental expression of the chicken intestinal MUC2 gene.* PLoS One, 2013. **8**(1): p. e53781.
322. Achanta, M., et al., *Tissue expression and developmental regulation of chicken cathelicidin antimicrobial peptides.* J Anim Sci Biotechno, 2012. **3**(1): p. 1-7.
323. Catherine L J Brown, S.J.M.Z., Tony Montina, G Douglas Inglis, *Corticosterone-mediated physiological stress alters liver, kidney, and breast tissue metabolomic profiles in chickens.* (Submitted to Poultry Science, June 2021).

Appendix A: Supplemental material for chapter 2

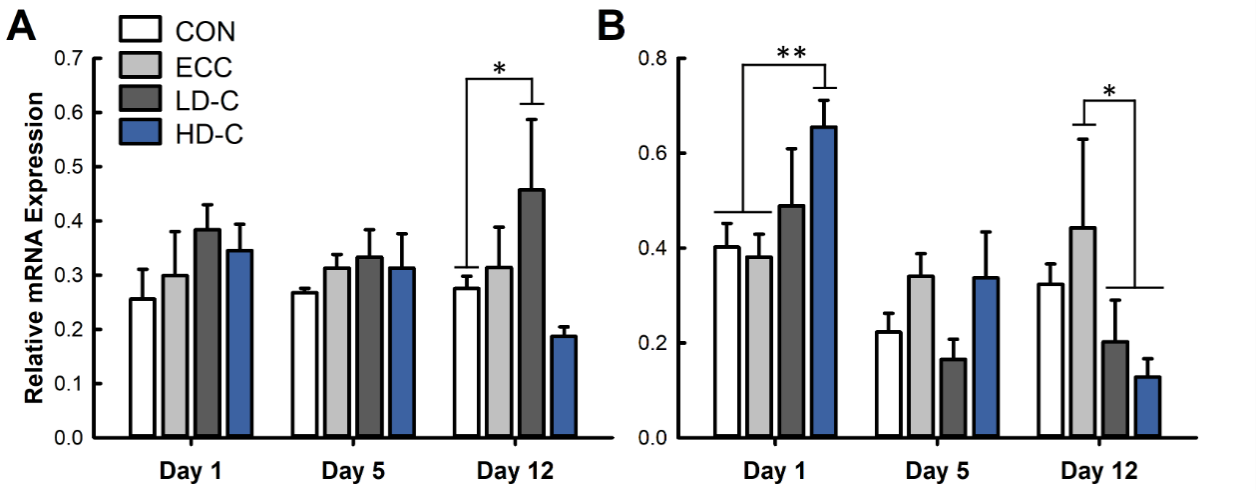

Figure S1.1 Effect of CORT treatment on *GLUT2* mRNA expression. Birds were administered standard drinking water (CON), 0.2% ethanol drinking water (ECC), 10 mg/L of CORT (LD-C), or 30 mg/L of CORT (HD-C); birds were euthanized and sampled at 1, 5, or 12 days post continual treatment. (A-B) relative mRNA gene expression of *GLUT2* in (A) liver and (B) jejunum. Bars represent means (n = 3) and vertical lines associated with bars represent standard error of the mean. * $P < 0.050$ and ** $P < 0.010$.

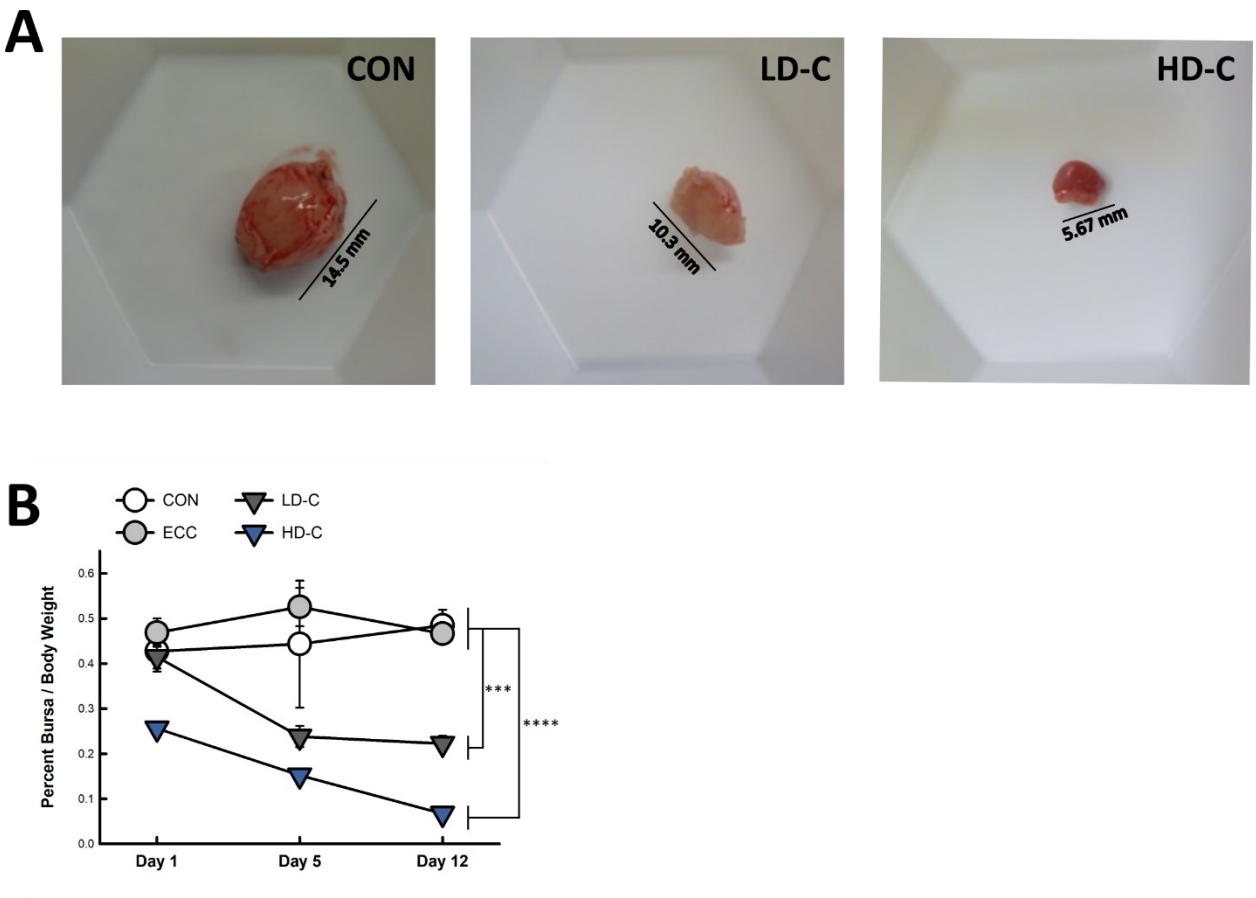

Figure S1.2 Corticosterone treatment reduces size of bursa of Fabricius. Birds were administered standard drinking water (CON), 0.2% ethanol drinking water (ECC), 10 mg/L of CORT (LD-C), or 30 mg/L of CORT (HD-C); birds were euthanized and sampled at 1, 5, or 12 days post continual treatment. (A) gross morphology of bursa of Fabricius after 12 days of CORT administration. (B) relative size of bursa of Fabricius to body weight. Markers represent mean (n = 3) and vertical lines associated with markers represent standard error of the mean. Markers without vertical lines indicates marker is obscuring the standard error of mean. *** $P < 0.001$ and **** $P < 0.0001$.

Table S1.1. List of primers used for mRNA gene expression

Name	Abbreviation	Sequence 5' to 3'		Product Size	Reference Sequence	Source
Fatty Acid Synthase	*FAS*	Forward	CAACAGCCAGCTTGGAATGG	161	NM_205155.2	This Study
		Reverse	CTGTGGTTCTCAGGGGCTTT			
Acetyl-CoA Carboxylase	*ACC*	Forward	CGTGAGGAGCCCATTCACAT	171	NM_205505.1	This Study
		Reverse	TGGAAATTCCCTCTTCTGTGC			
Malic Enzyme	*ME*	Forward	AATACACAGAGGGACGTGGC	121	NM_204303.1	This Study
		Reverse	GCAACTCCAGGGAACACGTA			
Sterol Regulatory Element Binding Factor	*SREBF*	Forward	CAGAAGAGCAAGTCCCTCAAG	129	NM_204126.2	This Study
		Reverse	GGAGCCTACATCCGAGGG			
Apolipoprotein B	*APOB*	Forward	AGCCAAACTAAGTGGACTGC	100	NM_001044633.1	This Study
		Reverse	GATCCGGCCTTCACTTTTCA			
Apolipoprotein C-III	*APOC3*	Forward	CAAGCCAGGAAATGGCTGTC	184	NM_001302127.1	This Study
		Reverse	GACAAAAGGGAAACGGTGCT			
Microsomal Triglyceride Transferase Protein	*MTTP*	Forward	AAACTGGAACTGCAATCGGT	101	NM_001109784.2	This Study
		Reverse	GGCATGGAAACGTAACTGGA			
Glucose Transporter 2	*GLUT2*	Forward	GGAGGCCAAAAAGAGTTTGAAG	105	NM_207178.1	This Study
		Reverse	ACTCTCTTTTCACTCGCAGC			
Serum Amyloid A1	*SAA1*	Forward	TGGGTGAAGTGGTGGACAAA	180	XM_003641328.3	This Study
		Reverse	CTGGACTATCTGAGCGGACT			
Ceruloplasmin	*CP*	Forward	GGGCTTAGGGTCAGAAGTCG	147	XM_015291853.1	This Study
		Reverse	TCAAAAATTCCTTCAGAATCAGGCT			
Transferrin	*TF*	Forward	TACTTCAGCGAGGGTTGTGC	148	NM_205304.1	This Study
		Reverse	CCAGACACCGTAAAGCTCCG			
Interleukin 1β	*IL1β*	Forward	TGCCTGCAGAAGAAGCCTCG	137	NM_204524.1	This Study
		Reverse	CTCCGCAGCAGTTTGGTCAT			
Interleukin 6	*IL6*	Forward	AGGACGAGATGTGCAAGAAG	176	NM_204628.1	This Study
		Reverse	CATTTCTCCTCGTCGAAGCC			
Transforming Growth Factor β2	*TGFβ2*	Forward	CCATCTACAACAGCACCAGGG	157	NM_001031045.3	This Study
		Reverse	TAGCTTGGTGGGATGGCATTTTC			
β-Actin	*BA*	Forward	CTCTGACTGACCGCGTTACT	172	NM_205518.1	This Study
		Reverse	TACCAACCATCACACCCTGAT			
Tata-Box Binding Protein	*TBP*	Forward	GTTCCCTGTGTCGCTTGC	147		PMID: 27685470
		Reverse	TAGCCCGATGATGCCGTAT			

Appendix B: Supplemental material for chapter 3

Table S2.1 Project metadata

Sample_ID	Biosample_Accession	Sample_Type	Time	Age of Bird	Stress_Treatment	Replicate	Bioproject_Accession
J-1A	SAMN15618862	Jejunum-Ileum Digesta	Day 1	15-days-old	Control	1	PRJNA647907
J-2A	SAMN15618863	Jejunum-Ileum Digesta	Day 5	19-days-old	Control	1	PRJNA647907
J-3A	SAMN15618864	Jejunum-Ileum Digesta	Day 12	26-days-old	Control	1	PRJNA647907
J-4A	SAMN15618865	Jejunum-Ileum Digesta	Day 1	15-days-old	Ethanol Control	1	PRJNA647907
J-5A	SAMN15618866	Jejunum-Ileum Digesta	Day 5	19-days-old	Ethanol Control	1	PRJNA647907
J-6A	SAMN15618867	Jejunum-Ileum Digesta	Day 12	26-days-old	Ethanol Control	1	PRJNA647907
J-7A	SAMN15618868	Jejunum-Ileum Digesta	Day 1	15-days-old	10 mg/L CORT	1	PRJNA647907
J-8A	SAMN15618869	Jejunum-Ileum Digesta	Day 5	19-days-old	10 mg/L CORT	1	PRJNA647907
J-9A	SAMN15618870	Jejunum-Ileum Digesta	Day 12	26-days-old	10 mg/L CORT	1	PRJNA647907
J-10A	SAMN15618871	Jejunum-Ileum Digesta	Day 1	15-days-old	30 mg/L CORT	1	PRJNA647907
J-11A	SAMN15618872	Jejunum-Ileum Digesta	Day 5	19-days-old	30 mg/L CORT	1	PRJNA647907
J-12A	SAMN15618873	Jejunum-Ileum Digesta	Day 12	26-days-old	30 mg/L CORT	1	PRJNA647907
J-1B	SAMN15618874	Jejunum-Ileum Digesta	Day 1	15-days-old	Control	2	PRJNA647907
1-2B	SAMN15618875	Jejunum-Ileum Digesta	Day 5	19-days-old	Control	2	PRJNA647907
J-3B	SAMN15618876	Jejunum-Ileum Digesta	Day 5	19-days-old	Control	3	PRJNA647907
J-4B	SAMN15618877	Jejunum-Ileum Digesta	Day 1	15-days-old	Ethanol Control	2	PRJNA647907
J-5B	SAMN15618878	Jejunum-Ileum Digesta	Day 5	19-days-old	Ethanol Control	2	PRJNA647907
J-6B	SAMN15618879	Jejunum-Ileum Digesta	Day 5	19-days-old	Ethanol Control	3	PRJNA647907
J-7B	SAMN15618880	Jejunum-Ileum Digesta	Day 1	15-days-old	10 mg/L CORT	2	PRJNA647907
J-8B	SAMN15618881	Jejunum-Ileum Digesta	Day 5	19-days-old	10 mg/L CORT	2	PRJNA647907
J-9B	SAMN15618882	Jejunum-Ileum Digesta	Day 5	19-days-old	10 mg/L CORT	3	PRJNA647907
J-10B	SAMN15618883	Jejunum-Ileum Digesta	Day 1	15-days-old	30 mg/L CORT	2	PRJNA647907
J-11B	SAMN15618884	Jejunum-Ileum Digesta	Day 5	19-days-old	30 mg/L CORT	2	PRJNA647907
J-12B	SAMN15618885	Jejunum-Ileum Digesta	Day 5	19-days-old	30 mg/L CORT	3	PRJNA647907
J-1C	SAMN15618886	Jejunum-Ileum Digesta	Day 1	15-days-old	Control	3	PRJNA647907
J-2C	SAMN15618887	Jejunum-Ileum Digesta	Day 12	26-days-old	Control	2	PRJNA647907
J-3C	SAMN15618888	Jejunum-Ileum Digesta	Day 12	26-days-old	Control	3	PRJNA647907
J-4C	SAMN15618889	Jejunum-Ileum Digesta	Day 1	15-days-old	Ethanol Control	3	PRJNA647907
J-5C	SAMN15618890	Jejunum-Ileum Digesta	Day 12	26-days-old	Ethanol Control	2	PRJNA647907
J-6C	SAMN15618891	Jejunum-Ileum Digesta	Day 12	26-days-old	Ethanol Control	3	PRJNA647907
J-7C	SAMN15618892	Jejunum-Ileum Digesta	Day 1	15-days-old	10 mg/L CORT	3	PRJNA647907
J-8C	SAMN15618893	Jejunum-Ileum Digesta	Day 12	26-days-old	10 mg/L CORT	2	PRJNA647907
J-9C	SAMN15618894	Jejunum-Ileum Digesta	Day 12	26-days-old	10 mg/L CORT	3	PRJNA647907
J-10C	SAMN15618895	Jejunum-Ileum Digesta	Day 1	15-days-old	30 mg/L CORT	3	PRJNA647907
J-11C	SAMN15618896	Jejunum-Ileum Digesta	Day 12	26-days-old	30 mg/L CORT	2	PRJNA647907
J-12C	SAMN15618897	Jejunum-Ileum Digesta	Day 12	26-days-old	30 mg/L CORT	3	PRJNA647907
C-1A	SAMN15618898	Cecum Digesta	Day 1	15-days-old	Control	1	PRJNA647907
C-2A	SAMN15618899	Cecum Digesta	Day 5	19-days-old	Control	1	PRJNA647907
C-3A	SAMN15618900	Cecum Digesta	Day 12	26-days-old	Control	1	PRJNA647907
C-4A	SAMN15618901	Cecum Digesta	Day 1	15-days-old	Ethanol Control	1	PRJNA647907
C-5A	SAMN15618902	Cecum Digesta	Day 5	19-days-old	Ethanol Control	1	PRJNA647907
C-6A	SAMN15618903	Cecum Digesta	Day 12	26-days-old	Ethanol Control	1	PRJNA647907
C-7A	SAMN15618904	Cecum Digesta	Day 1	15-days-old	10 mg/L CORT	1	PRJNA647907

C-8A	SAMN15618905	Cecum Digesta	Day 5	19-days-old	10 mg/L CORT	1	PRJNA647907
C-9A	SAMN15618906	Cecum Digesta	Day 12	26-days-old	10 mg/L CORT	1	PRJNA647907
C-10A	SAMN15618907	Cecum Digesta	Day 1	15-days-old	30 mg/L CORT	1	PRJNA647907
C-11A	SAMN15618908	Cecum Digesta	Day 5	19-days-old	30 mg/L CORT	1	PRJNA647907
C-12A	SAMN15618909	Cecum Digesta	Day 12	26-days-old	30 mg/L CORT	1	PRJNA647907
C-1B	SAMN15618910	Cecum Digesta	Day 1	15-days-old	Control	2	PRJNA647907
C-2B	SAMN15618911	Cecum Digesta	Day 5	19-days-old	Control	2	PRJNA647907
C-3B	SAMN15618912	Cecum Digesta	Day 5	19-days-old	Control	3	PRJNA647907
C-4B	SAMN15618913	Cecum Digesta	Day 1	15-days-old	Ethanol Control	2	PRJNA647907
C-5B	SAMN15618914	Cecum Digesta	Day 5	19-days-old	Ethanol Control	2	PRJNA647907
C-6B	SAMN15618915	Cecum Digesta	Day 5	19-days-old	Ethanol Control	3	PRJNA647907
C-7B	SAMN15618916	Cecum Digesta	Day 1	15-days-old	10 mg/L CORT	2	PRJNA647907
C-8B	SAMN15618917	Cecum Digesta	Day 5	19-days-old	10 mg/L CORT	2	PRJNA647907
C-9B	SAMN15618918	Cecum Digesta	Day 5	19-days-old	10 mg/L CORT	3	PRJNA647907
C-10B	SAMN15618919	Cecum Digesta	Day 1	15-days-old	30 mg/L CORT	2	PRJNA647907
C-11B	SAMN15618920	Cecum Digesta	Day 5	19-days-old	30 mg/L CORT	2	PRJNA647907
C-12B	SAMN15618921	Cecum Digesta	Day 5	19-days-old	30 mg/L CORT	3	PRJNA647907
C-1C	SAMN15618922	Cecum Digesta	Day 1	15-days-old	Control	3	PRJNA647907
C-2C	SAMN15618923	Cecum Digesta	Day 12	26-days-old	Control	2	PRJNA647907
C-3C	SAMN15618924	Cecum Digesta	Day 12	26-days-old	Control	3	PRJNA647907
C-4C	SAMN15618925	Cecum Digesta	Day 1	15-days-old	Ethanol Control	3	PRJNA647907
C-5C	SAMN15618926	Cecum Digesta	Day 12	26-days-old	Ethanol Control	2	PRJNA647907
C-6C	SAMN15618927	Cecum Digesta	Day 12	26-days-old	Ethanol Control	3	PRJNA647907
C-7C	SAMN15618928	Cecum Digesta	Day 1	15-days-old	10 mg/L CORT	3	PRJNA647907
C-8C	SAMN15618929	Cecum Digesta	Day 12	26-days-old	10 mg/L CORT	2	PRJNA647907
C-9C	SAMN15618930	Cecum Digesta	Day 12	26-days-old	10 mg/L CORT	3	PRJNA647907
C-10C	SAMN15618931	Cecum Digesta	Day 1	15-days-old	30 mg/L CORT	3	PRJNA647907
C-11C	SAMN15618932	Cecum Digesta	Day 12	26-days-old	30 mg/L CORT	2	PRJNA647907
C-12C	SAMN15618933	Cecum Digesta	Day 12	26-days-old	30 mg/L CORT	3	PRJNA647907

Table S2.2 Bird body weight gain[#]

	Average cumulative weight gain (g)		
	Day 0 to Day 1 (n=9)	**Day 1 to Day 5 (n=6)**	**Day 5 to Day 12 (n=3)**
CON	10.7	45.2	87.1
ECC	9.6	44.4	76.5
LDC	4.7**	16.0 ***	44.7*
HDC	4.9**	8.6***	48.4*

* P < 0.050; ** P < 0.010: *** P < 0.001 in comparison to CON and ECC treatments

Data has been previously published in a different format. Bird body weight was previously reported as daily bird weight over experimental period in Zaytsoff, S.J.M.; Brown, C.L.J.; Montina, T.; Metz, G.A.S.; Abbott, D.W.; Uwiera, R.R.E.; Inglis, G.D. Corticosterone-mediated physiological stress modulates hepatic lipid metabolism, metabolite profiles, and systemic responses in chickens. Sci Rep 2019, 9, 19225, doi:10.1038/s41598-019-52267.

Appendix C: Supplemental material for chapter 4

Table S3.1 Feed ingredients for 21% poultry starter diet.

Ingredient List	Minerals	Vitamins
Wheat	Copper Sulfate	Vitamin A
Soybean meal	Ethylenediamine dihydroiodide	Vitamin D
Canola meal	Manganese Sulfate	Vitamin E
Corn distillers	Sodium Selenite	Vitamin K
Faba beans	Zinc Oxide	Thiamine monohydrate
L-Lysine HCl	Copper Proteinate	Pryidoxine
Enzyme	Manganese Proteinate	Calcium pantothenate
Choline chloride	Selenium Yeast	Niacin
ALIMET	Zinc Proteinate	Riboflavin
Limestone		Biotin
		Folic acid
		Vitamin B12
		Ethoxyquin

Table S3.2 List of primers used for qPCR and mRNA gene expression

Name	Abbreviation	Sequence 5' to 3'		Product Size	Reference Sequence	Source
C. perfringens (16S)	*CP1.2*	Forward	AAAGATGGCATCATCATTCAAC	283		PMID: 12399288
		Reverse	TACCGTCATTATCTTCCCCAAA			
NetB Toxin	*NetB*	Forward	AAATATACTTCTAGTGATACCGCTTCACA	78		PMID: 20457789
		Reverse	GAGGATCTTCAATAAATGTTCCACTTAA			
Toll-like receptor 2A	*TLR2A*	Forward	CAGCACAAGAGGCGTTCA	100	NM_204278.1	This Study
		Reverse	AACATTTTGGTGTAGCTGAGATG			
Toll-like receptor 15	*TLR15*	Forward	ACTAGAACCTGCCTGCCAAC	102	NM_001037835.1	This Study
		Reverse	AGCATCAGGAGAGATTGCCC			
Claudin 3	*CLDN3*	Forward	GGGATTTCTACAACCCGCTG	200	NM_204202.1	This Study
		Reverse	CTTGTCGTAGCTGGTAACGG			
Claudin 5	*CLDN5*	Forward	GGGGACTAAATGCAGAGACC	128	NM_204201.1	This Study
		Reverse	ATAGCCTAAGCATCACGAGC			
Occludin	*OCLN*	Forward	TGAATGCACCCACTGAGTGTT	99	NM_205128.1	This Study
		Reverse	CCAGAGGTGTGGGCCTTAC			
Mucin 2B	*MUC2B*	Forward	ATTGTGGTAACACCAACATTCATC	135		PMID: 23349743
		Reverse	CTTTATAATGTCAGCACCAACTTCTC			
Mucin 5AC	*MUC5AC*	Forward	TCCACCAGCTTCCAAATCCC	79	XM_015286693.1	This Study
		Reverse	GGGGTTGCCAGCCTTTACTT			
Interleukin 1β	*IL1*β	Forward	TGCCTGCAGAAGAAGCCTCG	137	NM_204524.1	This Study
		Reverse	CTCCGCAGCAGTTTGGTCAT			
Interleukin 2	*IL2*	Forward	TAACTGGGACACTGCCATGA	93	NM_204153.1	This Study
		Reverse	GATAGAGATGCTCCATAAGCTGT			
Interleukin 18	*IL18*	Forward	GTGAAGAGATCGCTGTGTGT	80	NM_204608.1	This Study
		Reverse	ATCGCATTCCAGCTCATCATC			
Interferon γ	*IFN*γ	Forward	GCTCCCGATGAACGACTTGA	121	NM_205149.1	This Study
		Reverse	GCATCTCCTCTGAGACTGGC			
Interleukin 6	*IL6*	Forward	AGGACGAGATGTGCAAGAAG	176	NM_204628.1	This Study
		Reverse	CATTTCTCCTCGTCGAAGCC			
Transforming Growth Factor β2	*TGF*β	Forward	CCATCTACAACAGCACCAGGG	157	NM_001031045.3	This Study
		Reverse	TAGCTTGGTGGGATGGCATTTTC			
Colony Stimulating Factor 3	*G-CSF*	Forward	AACTTATTTGTGCCCCCACG	84	NM_205279.1	This Study
		Reverse	ATACAGTACAAAACGCCGCC			
β-Actin	*BA*	Forward	CTCTGACTGACCGCGTTACT	172	NM_205518.1	This Study
		Reverse	TACCAACCATCACACCCTGAT			
Tata-Box Binding Protein	*TBP*	Forward	GTTCCCTGTGTCGCTTGC	147		PMID: 27685470
		Reverse	TAGCCCGATGATGCCGTAT			

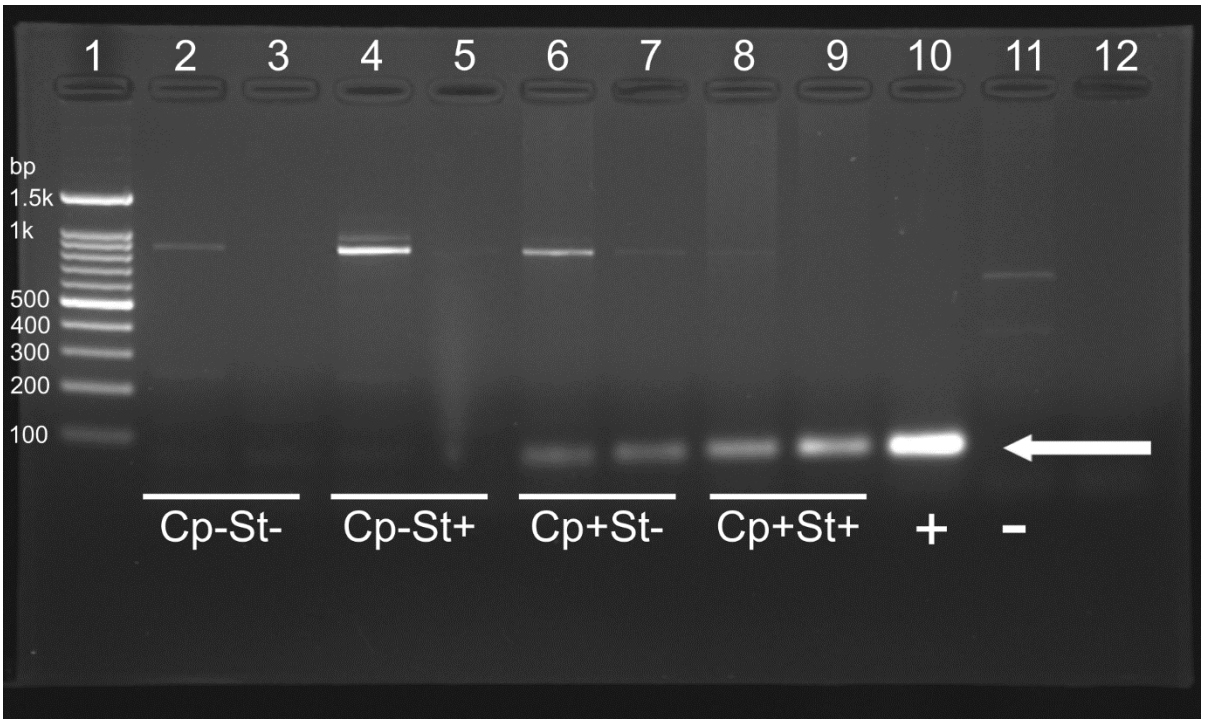

Figure S3.1 A 2% agarose gel of NetB gene PCR product (78 bp). Fecal samples were collected 24 hr post inoculation with control broth or CP1 *C. perfringens* (NetB toxin positive), DNA was extracted, and subjected to conventional PCR. Lane 1: SMOBIO 100 bp DNA Ladder; Lane 2/3: PCR product of negative control birds (Cp-St-); Lane 4/5: PCR product of birds receiving 20 mg/L corticosterone (Cp-St+); Lane 6/7: PCR product of birds receiving *C. perfringens* inoculation (Cp+St-); Lane 8/9: PCR product of birds receiving both *C. perfringens* inoculation and 20 mg/L corticosterone (Cp+St+); Lane 10: PCR of positive control containing DNA isolated from CP1 *C. perfringens* strain; Lane 11: PCR of negative control containing DNA isolated from confirmed NetB negative *C. perfringens*; and Lane 12: PCR of negative control water blank. Positive bands for NetB gene amplification are seen only in samples obtained from Cp+St- and Cp+St+ treatment groups.

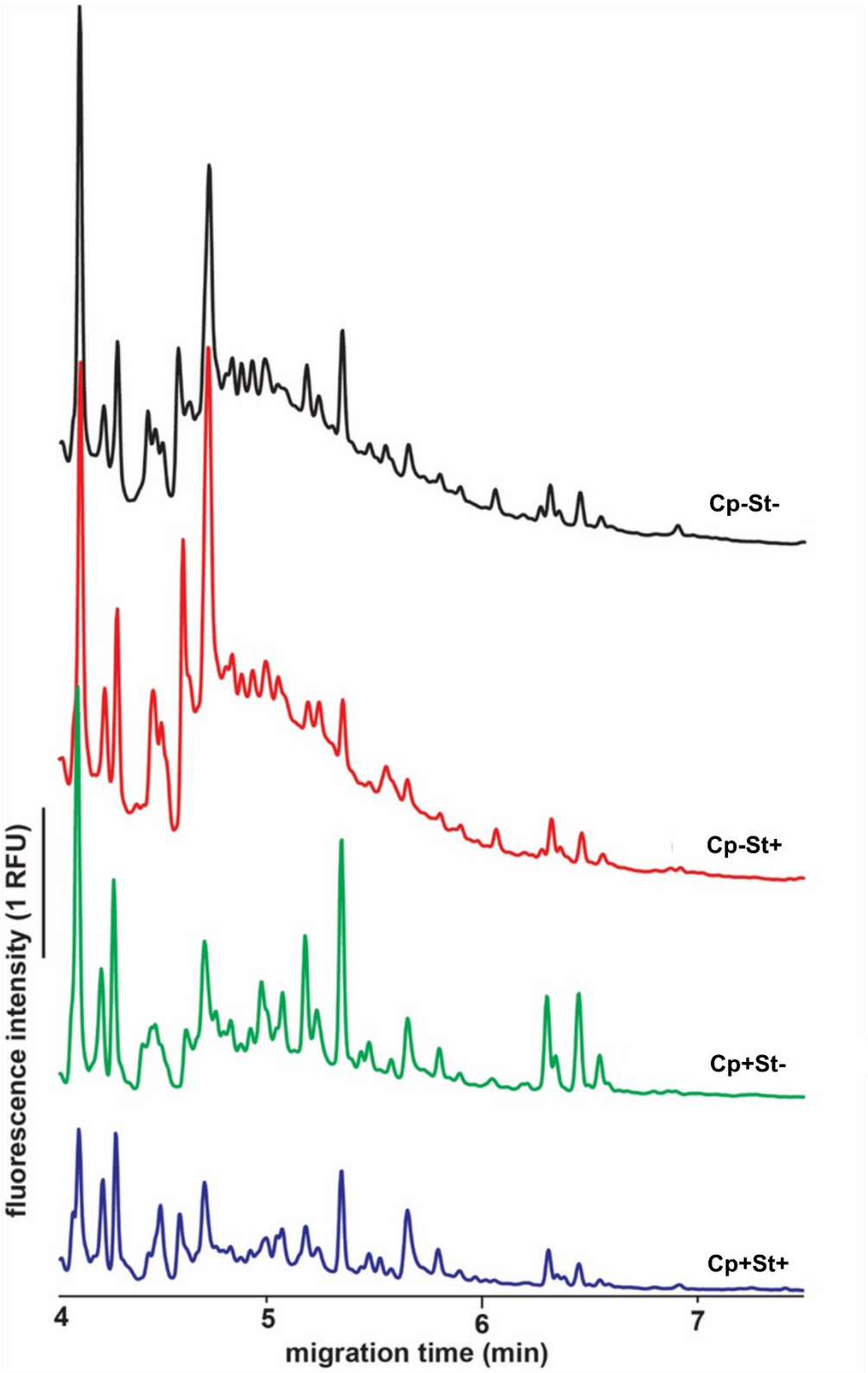

Figure S3.2 Representative capillary electrophoresis electropherograms of APTS-labelled *O*-glycans derived from intestinal mucus of chickens in the four treatment groups.

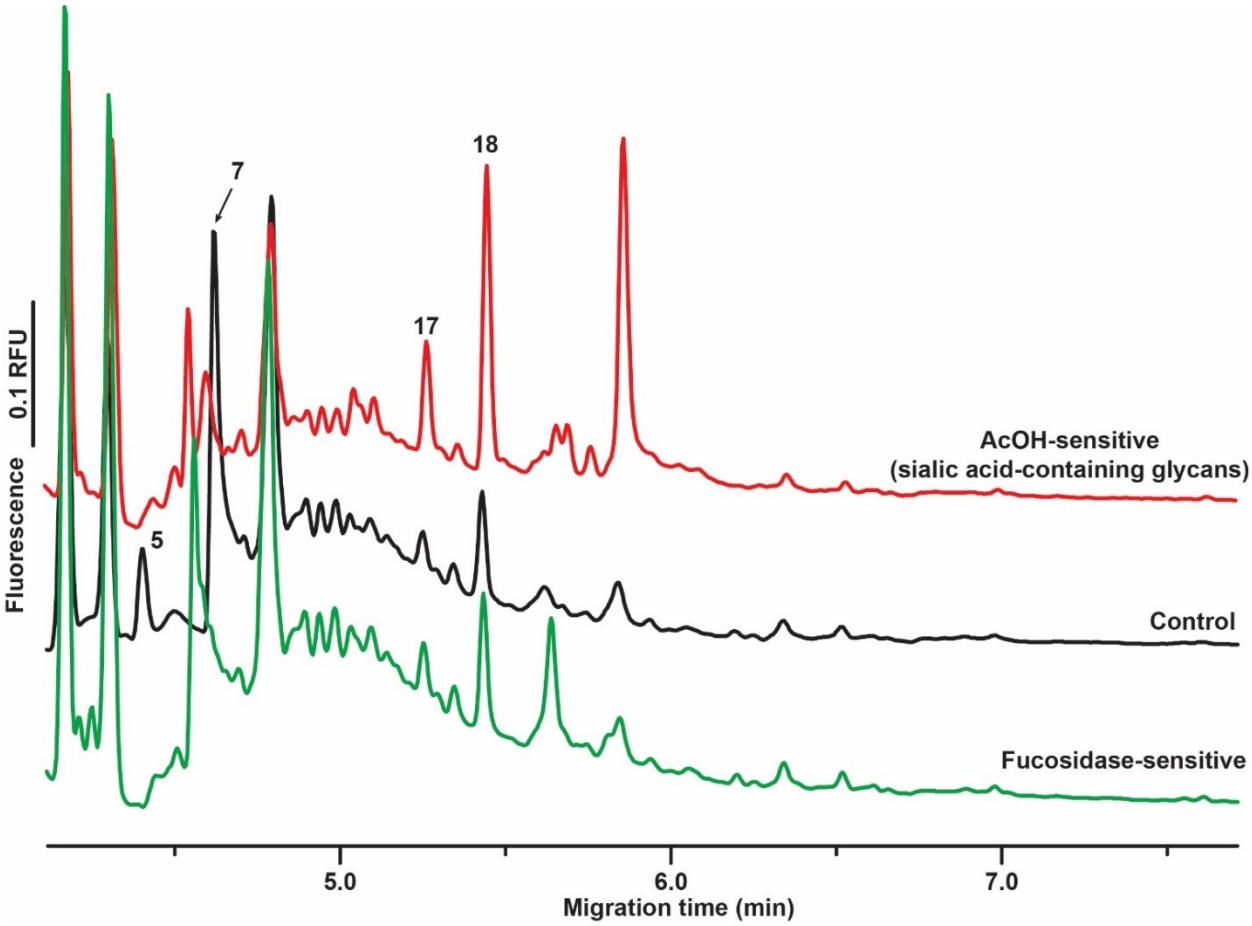

Figure S3.3 Representative capillary electrophoresis electropherograms indicating sialic acid and fucose-containing *O*-glycans as inferred after their loss upon acetic acid (AcOH) or fucosidase-treatments, respectively. Selected glycans are numbered as described in Figure 4.3A.

Appendix D: Refinement of corticosterone model and induction of necrotic enteritis model

Table S4.1 Modifications to corticosterone administration and development of stress-induced necrotic enteritis (NE) model.

Procedural Modification	Rationale	Benefit	Considerations
Transition from layer to broiler chickens	Examine differences in NE severity between layer and broiler chickens	Applicable to broilers, which are severely impacted by NE	Did not ascertain why broiler birds were more susceptible to stress-induced NE
Changes to the dose of corticosterone used	Optimize dose to promote disease susceptibility	Attempt to better mimic a physiological stress response that occurs in production when multiple concurrent stressors occur	Verifyication of the corticosterone administration relative to production stressors, and the identification of biomarkers of stress and disease are currently under investigation
Water versus in-feed corticoster-one administration	Remove exposure to ethanol in drinking water, and subsequent impacts on ethanol on the host	In-feed administration eliminates bird exposure to ethanol	The comsumption of corticsterone between water and feed varies