8-2022

Intestinal Microbiota Analysis of Broiler Chickens under Necrotic Enteritis Challenge and Tributyrin Supplementation

Taylor Nicole McKinney
University of Arkansas, Fayetteville

Intestinal Microbiota Analysis of Broiler Chickens under Necrotic Enteritis Challenge and Tributyrin Supplementation

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science in Animal Science

by

Taylor McKinney
University of Arkansas
Bachelor of Science in Animal Science, 2018

August 2022
University of Arkansas

This thesis is approved for recommendation to the Graduate Council.

Jiangchao Zhao, PhD
Thesis Director

Kenneth P. Coffey, PhD
Committee Member

Xiaolun Sun, PhD
Committee Member

ABSTRACT

Poultry is a staple protein source for most of the planet. Until recently, antibiotic growth promoters (AGPs) were used to prevent illnesses in commercial chicken production. Currently, this is not possible due to regulations and consumer concern, but without such a preventative, diseases like necrotic enteritis (NE) have reemerged, posing a threat to bird health, and ultimately, our food source. Necrotic enteritis is a severe gastrointestinal disease caused by the gram-positive pathogen, *Clostridium perfringens.* Clinical features of this disease are diarrhea, intestinal lesions, and death, with a high transmission rate. In a subclinical form, growth performance is diminished and is the primary cause of economic loss to producers. Butyrate substances have been introduced to replace AGPs. Studies show these substances appear to relieve intestinal damage that is caused by NE. The relationship between gut health and gut microbiota community structure is well established in human studies. It is expected that animals are affected by their gut microbiota composition similarly. It is unclear whether the butyrate treatment influences the chicken GI microbiota composition or if such a change would help explain the mechanisms that improve intestinal lesions in birds affected by NE. By using 16S rRNA High-Throughput Next Generation (HTNG) amplicon gene sequencing, we compared the microbial composition of the cecum and ileum of birds from three different groups: T1, nonmedicated, unchallenged with *C. perfringens* (negative control group), T2, nonmedicated, challenged with *C. perfringens* (positive control group), and T6, treated with butyrin (Butyrin SR130, Perstorp) in the feed at 0.5kg/metric ton from day 0 to day 14 and at 0.25 kg/metric ton from day 14 to 20 (variable dose) and challenged with *C. perfringens* (Hofacre, et al., 2020). The objective of this study was 1) to assess the efficacy using gut microbial communities as a novel measure of NE in broiler chickens, and 2) to assess the efficacy of a butyrate treatment for

NE in chickens. The results indicated no significant effect on beta diversity of microbial community structure among the three treatment groups. The disease challenge in groups T2 and T6 was observable and significant, yet the microbial composition and abundance of *C. perfringens* were visually indistinguishable among the three groups of birds. Random Forest analysis identified some enriched features in the T1 and T6 groups that were rarely present in the T2 group of the ileum and cecum. This thesis explores the potential explanations for the lack of microbial diversity between unchallenged birds, and birds intentionally inoculated with a known pathogen, as well as a further look into the enriched features identified by Random Forest. These features may play a small role in the recovery of NE through tributyrin treatment that additional research could explain.

ACKNOWLEDGEMENTS

Foremost, I would like to express my sincere gratitude to my graduate advisor, Dr. Jiangchao Zhao, for his continuous support, patience, and immense knowledge, and for allowing me the opportunity to further my academic career under his guidance. I would also like to thank my committee members, Dr. Ken Coffey, Dr. Shane Gadberry, and Dr. Xiaolun Sun, as well as department head, Dr. Michael Looper, for the expertise they each brought to this project and whose support was greatly appreciated. Thank you, Dr. Coffey, for pushing me to finish this project and for your genuine kindness throughout my time not only as a graduate student but also as a very nervous undergraduate transfer student.

I thank my fellow lab mates and colleagues, Jianmin Chai, Xiaofan Wang, Dr. Tsung Cheng Tsai, and Robert Story for answering many, many questions and performing some of the most vital work for this project. It would not have been possible without you all.

I would like to thank my friend, Sarah Shelby, for her continuous support during what have been some of the most difficult years of my life. Your friendship has helped me to navigate not only the graduate program but also my life. I could not have done this without having you in my corner every step of the way.

DEDICATION

For AnnaBelle

"The cow is the foster mother of the human race. From the day of the ancient Hindoo to this time have the thoughts of men turned to this kindly and beneficent creature as one of the chief sustaining forces of human life." - W.D. Hoard

TABLE OF CONTENTS

CHAPTER I.

Review of Literature

1. Introduction

Decades of advancement in science and technology have been utilized by the agriculture and poultry industries to produce more food now than ever before. The birth of the broiler industry in the 1920's consisted of a 16 square-foot, 500-bird capacity barn (Godley et. al., 2020), heated by a coal stove, and the management of feeding and watering those birds was conducted by hand. Modern poultry producers in the U.S. can adjust their 20,000-bird capacity broiler-house settings for temperature, humidity, lighting, and automated feeders and waterers as easily as operating a smartphone. In addition, our scientific understanding of heritable traits in the 1970's has allowed geneticists to breed quick-growing, large birds, ideal for meat consumption. What is more, we can calculate nutritional requirements and formulate diets that optimize feed efficiency and animal health for each stage of bird growth. Thanks to advances in veterinary science, we have a greater understanding of the diseases threatening our flocks, and until recently had nearly unbridled access to antibiotics for the treatment of many of those diseases. These advances have led to better animal health and some of the most sustainable management practices of conventional livestock production.

One of the most recent scientific innovations of meaningful application to poultry research and industry includes the ability to conduct a microbial census within a targeted biological location. These microscopic organisms include bacteria, viruses, and yeasts and vastly outnumber host gastric cells. Assessing the composition of the chicken's gut microbial community may be the next diagnostic tool for diagnosing and maintaining overall bird health. Much like improved understanding of animal breeding and nutrition propelled the poultry industry forward, the effects of 16S rRNA gene sequencing techniques used in microbial research for disease control, broiler house-environment maintenance, growth performance, and feed efficiency, among other

factors, is likely to maintain this forward progress. Bird health is greatly affected by gastrointestinal health, and comprehensively the health of the gut microbiome. To best understand innovative microbiota research methods and their application to bird health, a look at the gastrointestinal tract of broiler chickens is essential.

2. Gastrointestinal Health of Broiler Chickens

2.1. Morphology and Physiology of Broiler Gastrointestinal System

The GI tract of chickens begins at the beak. This is the site of ingestion and passage of food to the upper esophagus. The upper esophagus transports food to the crop for storage unless the bird's stomach is empty. If empty, food will bypass the crop, entering the lower esophagus for transport to the proventriculus and gizzard. The proventriculus, or glandular stomach, is comprised of oxynticopeptic cells which secrete digestive enzymes such as pepsinogen, as well as hydrochloric acid, and mucous (Denbow, 2015). These enzymes begin to break down the soluble components of the ingested food and moisten it into a soft bolus before transferring it to the gizzard. The gizzard functions as the main masticatory organ, collecting grit and smashing the bolus apart into tiny particles, increasing its surface area for enzymatic digestion. Once broken down physically and the chemical digestion process has sufficiently begun, food particles enter the small intestine at the duodenum. Duodenal, jejunal and ileal villi, and microvilli, with the help of the pancreas and liver, secrete protein-, fat-, and carbohydrate-specific enzymes for further nutrient digestion and the process of nutrient absorption begins. Any remaining small or soluble undigested food particles enter one of the two cecal tubes – the main sites of microbial fermentation (Svihus et al., 2013). After roughly 24 hours, the remaining undigested food particles enter the large intestine, the primary site of water reabsorption.

2.2. Enteric Disease Control without Antibiotics

Combating enteric disease has historically been challenging for the poultry industry, even more so with the recent removal of antibiotics in a consumer-driven and evolving market. In recent years, concerns about the overuse of antibiotics in the agricultural industry have grown, followed by a reduction in the commercial use of antibiotics and antibiotic growth promoters (AGP). The diseases these antibiotics formerly treated and/or prevented remain a threat to bird health in the absence of antibiotics.

Antibiotics have been used in animal production not only for the treatment of disease but as AGP to improve growth rate and feed efficiency (Costa et al., 2017). It is the latter of the two uses that has spiked concern due to fear of creating antibiotic-resistant "superbugs". The threat of antibiotic-resistant pathogens also poses other problems to human medicine and our food chain (Fasina et al., 2016). Additionally, it has been demonstrated that antibiotics have a significant effect on the reduction of commensal gut bacteria, provoking a balance shift and risking pathogenic overgrowth (Kogut, 2019). An alternative to antibiotics must be identified, or the health of the birds and the efficient reputation of the poultry industry are at risk.

2.3. Necrotic Enteritis Etiology

The global poultry industry currently suffers an estimated USD 2 Billion in economic loss (Latorre et al., 2018) each year solely to the severe gastroenteric disease, NE. Necrotic enteritis is induced, simply, by disequilibrium of environment and/or diet (Antonissen et al., 2016). The definitive organism responsible for NE disease is *Clostridium perfringens*, a Gram-positive, spore-forming, toxin-producing, rod-shaped anaerobe (Shojadoost et al., 2012). This bacterium is among the normal microbiota that inhabit the GI tract of chickens and humans, and in almost every aspect of a typical farm environment: soil, feces, and chicken litter, but it easily becomes

pathogenic under the right conditions (Williams, 2005; Wade et al., 2016). The natural clostridial burden varies from bird to bird and the age of the bird positively correlates with the clostridial burden (Williams, 2005), nevertheless, most incidences of NE in the poultry industry occur at around four weeks of age, before birds reach market size (Riaz et al., 2017). Fasina, 2016, found that under normal conditions, a healthy broiler's small intestine will usually contain around 10^4 CFU (colony forming units) of *C. perfringens* per gram of digesta. The presence of *C. perfringens* alone, however, is not enough to trigger clinical or subclinical NE onset (Shojadoost et al., 2012).

NE is commonly exacerbated by any one or a combination of the following: intercurrent coccidiosis infection, high protein diet, the presence or absence of certain bacteria within the bird's environment, and recently, the removal of antibiotic growth promoters which allow the proliferation of *C. perfringens* to exceed normal quantities (Collier et al., 2008). The intensity of this disease's associated microbial infection and *C. perfringens* various, and in some cases unidentified, virulence factors make it a complex disease that is still not well understood in the scientific community (Tamirat et al., 2017). Several predisposing factors which put birds at greater risk for contracting NE have been identified.

2.3.1 Predisposing Factors

i. Coccidiosis as a Predisposing Factor

A rivaling enteric disease in chickens, coccidiosis, is caused by various species of the protozoa *Eimeria*, commonly *E. maxima, E. acervulina,* and *E. tenella* (Shojadoost et al., 2012; López-Osorio et al., 2020). Each species of *Eimeria* installs a unique, site-specific, disease challenge, varying in the severity of symptoms, creating a coccidiosis infection dichotomy: clinical or subclinical. In broilers, a sub-clinical infection caused by *E. acervulina* or *E. maxima* tends to be

more chronic in nature, causing lesions along the duodenum and jejunum, and *E. maxima* may even cause lesions in the ileum (López-Osorio et al., 2020). *E. tenella* primarily attacks the ceca, causing acute GI distress and tissue damage, characterized by loose, bloody stools (De Gussem, 2007; López-Osorio et al., 2020). Because of the differences in severity and primary site of infection, certain *Eimeria* species are more likely to invoke the necessary conditions for the onset of NE and thus are commonly utilized for experimental induction of NE (Shojadoost et al., 2012).

For more than 40 years, ionophores, such as monensin, were used to control coccidiosis. Ionophores are natural substances that selectively transport ions across cell membranes that control coccidiosis by channeling Na+ into the coccidial sporozoites causing them to burst (Chapman et al., 2010). Because live vaccines are in use to protect chicks from coccidiosis, there has been a decline in the use of ionophores as a preventative for coccidiosis (Williams, 2005). Due to the live pathogens present in the vaccine, however, the use of the ionophore would disrupt the vaccine efficacy.

It has been demonstrated that an intercurrent coccidiosis infection is a leading cause of NE in broilers, primarily because the initial infection and damage to the host creates an ideal environment for *C. perfringens* colonization. Coccidiosis is considered a predisposing factor to NE in chickens partly due to the host's inflammatory immune response. Aimed to rid the body of coccidia by stimulating mucogenesis in the intestines, the resulting mucous serves as an ideal growth medium for *C. perfringens,* thus promoting the onset of NE (Collier et al., 2008). The birds in that study that were fed *Narisin*, an ionophore specifically meant to eliminate coccidia, exhibited reduced size of epithelial mucous-producing goblet cells and subsequently reduced NE lesions and *C. perfringens* colony forming units (CFUs) (Collier et al., 2008). Another reason

coccidiosis predisposes birds to NE is due to damage to intestinal cells and lining, giving rise to opportunistic clostridial infections (Williams, 2005). This intestinal damage results in the leakage of proteins and growth factors into the intestinal lumen where they are readily utilized by *C. perfringens* for proliferation (Shojadoost et al., 2012; Tamirat, et al., 2017). Although coccidiosis is a major predisposing factor, NE can still be induced by certain types of diets and other factors.

ii. Diet as a Predisposing Factor

Diet type affected the prevalence and severity of NE in broiler chickens in several studies. Diets consisting of cereal grains rye, wheat, barley, and oats, resulted in greater disease severity than corn-based diets fed to birds (Williams, 2005). It is believed that the addition of non-starch polysaccharides favors colonization of *C. perfringens* because this diet type increases digesta viscosity, slowing the passage rate and ultimately decreasing digestibility (Pan & Yu, 2013). Wheat and barley diets also contributed to *C. perfringens* proliferation when compared to corn diets in an *in vitro* digestion trial (Annett et al., 2002). Corn-based diets help prevent the growth and proliferation of *C. perfringens,* but the mechanisms that allow this diet to do so are still not greatly understood. It was suggested that enzymatic digestion of corn activates some unknown component(s) found within the corn kernel responsible for inhibition of *C. perfringens* proliferation, as found in the *in vitro* study (Annett et al., 2002). More work on the enzyme activation of corn in poultry diets is needed to understand one of the many mechanisms that may suppress NE.

Cereal grain diets are just one nutritional trigger for NE. Diets high in protein, particularly fishmeal, are another. High protein diets contribute to the onset of NE by providing *C. perfringens* with the nutrients needed to colonize the gut, increasing mucus secretion and

damaging intestinal mucosa, further weakening the bird's immune system (Xu et al., 2018). The added protein in broiler diets creates an amino acid-rich environment, feeding and compensating for *C. perfringens*'s lack of the necessary genes to conduct efficient amino acid biosynthesis (Antonissen et al., 2016).

It is not only an increase in dietary crude protein that predisposes birds to NE. A broiler diet's protein source serves as a better indicator of *C. perfringens* growth potential than dietary or crude protein level alone, and when the protein source is animal-derived, intestinal *C. perfringens* growth is statistically greater (Drew et al., 2004; Wilkie et al., 2005). In trial settings, a fishmeal-based diet and vaccination against coccidia, as mentioned previously, are commonly used to successfully induce NE in live birds.

iii. Stress as a Predisposing Factor

The temperature within the broiler house has been causally linked to the occurrence of NE and disruption of homeostasis in the bird. Both cold and heat stress have been found to correlate with increased prevalence of NE and increased *C. perfringens* counts. It has been suggested that cold stress induces immunosuppression and alterations in the microbiota community composition which create a convenient avenue for *C. perfringens* proliferation (Tsiouris et. al., 2015a). Heat stress is widely known to inhibit growth performance, by triggering a reduction in feed intake causing damage to the bird's immune system. Heat stress stimulates the activation of the hypothalamus-pituitary-adrenal axis and increases the production of glucocorticoids found in plasma, leading to cell-mediated and humoral immunosuppression (Tsiouris et al., 2018). It is also linked to impaired intestinal morphology, weakening the intestinal barrier function, and microbiota community dysfunction - all serious risks to NE outbreak.

There is conflicting evidence that heat stress predisposes birds to NE infection and yet, in some studies, the incidence of NE outbreak was more prevalent when taking place under colder conditions (Tsiouris et al., 2018). What is clear is that when birds are subjected to temperatures outside of a comfortable range, the body's stress signals lead to conditions where *C. perfringens* proliferation and NE are likely to follow. Proper management of the environmental conditions within the broiler house may reduce the onset of NE altogether when no other afflictions are presented concurrently.

iv. Dysbiosis as a Predisposing Factor

Improper diet, management style, exposure to other pathogens, and the onset of their subsequent diseases create potentially lethal situations for birds. Underlying the physical manifestation of increased morbidity and mortality are interactions between and among the microbes that are only beginning to be understood. We know that dysbiosis - the shifting microbial community characterized by deterioration of the lumen, pH fluctuations, and other unfavorable effects- rapidly supports *C. perfringens* colonization (Latorre et al., 2018). Seldom referred to in the literature, however, is the involvement of other known pathogenic bacteria as catalysts to NE. In 2018, researchers inoculated birds with a poultry isolate of *Salmonella* Typhimurium in combination with *E. maxima* to successfully induce a NE challenge model (Latorre et al., 2018). *Salmonella* is from the phylum *Proteobacteria* which is known for containing several opportunistic pathogens including *Escherichia, Salmonella,* and *Campylobacter (*Latorre et al., 2018). The current research suggests that any of these pathogens, and others, can be responsible for provoking and escalating *C. perfringens* growth and NE epidemics. At the least, without predisposing factors of diet, stress, intercurrent coccidiosis infection, or bacterial enteric disease, NE would not be the drain on animal welfare and economic loss that it has become.

Nevertheless, without antibiotics, we will face these issues under our current production and management standards.

2.4 Growth and Performance Losses During Necrotic Enteritis

Birds suffering from subclinical NE exhibit losses in performance traits including body weight gain (BWG), and enteric inflammation combined with mucosal permeability, or "leaky gut syndrome" (Latorre et al., 2018). Leaky gut demolishes the intestinal barrier function, making the animal susceptible to internal attack from pathogens and toxins normally inhabiting the intestinal lumen (Stewart et al., 2017; Latorre et al., 2018). Stress is one of the most common causes of leaky gut disease. Birds under stressful conditions release cortisol and harmful endotoxins, such as lipopolysaccharide, which are known to induce inflammatory cytokine production (Stewart et al., 2017). A recent study found that fluorescein isothiocyanate dextran (FITC-d), a measurement of enteric inflammation and mucosal permeability, leaked into the blood circulation in NE diseased birds, while under normal conditions, this signature of leaky gut syndrome was not present (Latorre et al., 2018). The result of birds under stress and experiencing leaky gut syndrome is reduced food intake which leads to reduced body weight gain. Because of the confined housing system and the gastrointestinal involvement of the disease, birds experiencing leaky gut because of NE spread the pathogen rapidly to other birds. Leaky gut syndrome is often lethal to broilers, so high mortality and morbidity are expected.

3. Gastrointestinal Microbiota of Broiler Chickens

3.1 A Brief History of Microbial Research

Until the early 2000s identifying bacteria and other microscopic organisms present in an environment was limited to cell culture (Wei et al., 2013). This process uses growth media and

specific induced environmental conditions to encourage the colonization of microbes taken from a given sample. Few intestinal microbes flourish on cell culture media (Lan, et al., 2002) and this technique cannot capture the interactions between microbial members in a natural community environment (Wei et al., 2013; Allali et al., 2017). Because of these limitations, this method of identification was replaced as quickly as possible with those more capable of accurately depicting the thriving, robust host microbiome as it naturally exists. Microbiota research as it is conducted today would not be possible without the use of DNA sequencing.

The earliest sequencing, "dideoxy sequencing" or "chain termination sequencing", is attributed to Fred Sanger. His methods were used to successfully decipher the protein sequence of insulin in the 1950s and later, in the 1960s, RNA sequences (Shendure et al, 2017). Using the now well-known Sanger sequencing methods, unknown genetic sequences are decoded by replicating the unknown sequence and exposing the sequence to DNA polymerase and a radioactive primer thus initiating a chain termination sequence. Then, gel electrophoresis is used to illuminate the chain termination fragments and the corresponding nucleotide position in the order of the sequence. The resulting band pattern across four lanes (one for each of the possible nucleotides) of a denaturing polyacrylamide gel reveals the previously unknown genetic sequence using the terminated fragments (Shendure et al, 2008). As great a success as Sanger sequencing proved to be, we now have access to faster, cheaper, and more reliable cell-sequencing technology, capable of providing results in a matter of hours.

3.1.1 Next Generation Gene Sequencing Techniques to Determine Phylogenetic Composition

The transition to second-generation, or next-generation, sequencing was preceded by molecular fingerprinting methods, including the Sanger method, which was more advanced and reliable

than basic cell-culture methods but did not provide the same level of diversity analysis that the modern method offers (Mohd Shaufi et al., 2015). Microbial profiling is no longer limited to low-accuracy, culture-dependent methods, but can now be performed via high-throughput next-generation sequencing (HT-NGS). This method is universally used for microbial research because it offers a faster, cheaper, and more in-depth coverage of the dynamic macrocosm within the human gut (Mohd Shaufi et al., 2015). Several sequencing platforms have been developed and commonly Illumina's MiSeq or HiSeq, and Roche's 454 GS FLX or 454 GS Junior are utilized for microbial research (Di Bella et al., 2013; Allali et al, 2017). The next-generation sequencing methods performed to determine the phylogenic microbial composition of an environment commonly involve targeted enrichment strategies to amplify and "read" specific regions of highly conserved bacterial DNA (Di Bella et al., 2013; Morey et al., 2013).

Deoxyribonucleic acid (DNA), simply, is the collection of nucleic acids that exist in paired chains in a living organism in a specific pattern. Certain portions of an organism's DNA are called genes. Genes have been defined as "a union of genomic sequences encoding a coherent set of potentially overlapping functional products" (Gerstein et al., 2007). In almost all bacteria and some archaea species, the 16S rRNA gene is shared among members and is commonly used to identify these organisms. Within the 16S rRNA gene are nine hypervariable sub-regions (V1-9), each evolving at their own pace and representing only a small fragment of the 16S genetic marker. To identify bacteria and create an image of the host's microbial community, researchers select the sub-region that provides the greatest coverage of microbial DNA in the target location of the host, based on known primer availability, fragment length, and region-associated sequence quality (Schloss, 2010).

It is well documented that there are nine (9) hypervariable regions of the 16S rRNA gene encoded by all bacteria and some archaea species that are suitable for amplification alone or in combination (Mohd Shaufi et al., 2015), but the different regions do not equally distinguish between bacterial species (Di Bella et al.,2013). In fact, due to the variability of sequence diversity among the nine hypervariable regions, no single region can distinguish among all bacteria (Chakravorty et al., 2007). The V3-V4 hypervariable region of 16S rRNA is the most widely used for taxonomic classification of gut microbiota (Darwish et al., 2021), but most researchers agree that the combination of V1-V4 regions is reliably accurate and suitable for meaningful bacterial classification within the chicken gut, though no universal standard currently exists (Kim, et al., 2011; Di Bella et al., 2013; Darwish et al., 2021). Johnson et al, 2019, argue that the best sub-region for classifying sequences belonging to the genera *Clostridium* is the V6-V9 region, but that study was not focused on classifying members of the chicken microbiota specifically. This study also argues the importance of adopting third-generation sequencing methods as the standard, which would allow for targeting of the entire hypervariable region of the 16S rRNA gene. Using only second-generation sequencing, this method was avoided partly because an assembly step was required and because an increase in sequence length increased the difficulty of gene assembly and rare taxa identification (Di Bella et al., 2013). Ballou et al., 2016 points out that the use of V4 sequencing primers provides microbial diversity results comparable with those of full-length 16S gene sequencing, which would likely negate the need to perform these extra steps. With the prospect of third-generation sequencing, we may see an increase in microbiota community compositional studies sequencing the entire hypervariable region (V1-V9), reducing the PCR and/or sequencing error (Johnson et al., 2019).

3.1.2 Data Analysis of 16S rRNA Sequences

Traditional methods of statistical analysis are not sufficient to conduct microbial ecology studies. The nature of the data collected during microbial gene sequencing and the bioinformatic processing of the sequences results in an overwhelming amount of raw data, or "metadata". Several software packages such as *Mothur* and *Qiime* (often referred to as bioinformatics pipelines) have been developed to process the mass amounts of biological data involved in microbiota research. The functions of these pipelines include the preparation of sequences directly from the sequencing platform (Illumina, Roche), Operational Taxonomic Unit (OTU) clustering, analysis of alpha and beta diversity, and data visualization, among others (Nilakanta et al., 2014). Not all software programs are created equally, as some programs offer features that may provide a cleaner inference of the microbiota due to differences in chimera or contaminant extraction, or variability in analysis options (Nilakanta et al., 2014). The differences among bioinformatics pipelines and similarly among the sequencing platforms are well documented, but it appears that the same biological conclusions can be drawn from chicken-gut samples regardless of the sequencing platform and/or the bioinformatics pipeline used (Allali et al., 2017). Across the literature, the programs *Mothur* and *Qiime* have stood out as the highest performing and most chosen for chicken GIT microbiota research (Nilakanta et al., 2014).

3.2 Broiler Chicken Ileum and Cecum Function as related to Microbial Characterization

Two sections of the broiler intestinal system, the ileum, and cecum are established in the literature as microbial cornucopias. These distinct segments of the GIT are commonly targeted for microbial research because of this, and the roles they perform in nutrient digestion and absorption (Clavijo & Flórez, 2018). The broiler chicken ileum is responsible for starch digestion, glucose, amino acid, lipid, and water absorption, and the reuptake of bile salts

(Denbow, 2015; Krogdahl, 1985). In addition to its role in nourishment, the entire small intestine is highly involved in immunoregulation, which provides overlap for microbial colonization selectivity. The mucus layer covering the intestinal epithelium acts as a barrier to pathogens attempting to enter the circulatory system but also serves as a substrate for commensal bacterial growth in the gut (Koutsos et al., 2006). The importance of the small intestine and its relationship to bird health cannot be overstated. Likewise, the microbial niche that develops in the ileum supports the capacity of the ileum to carry out its functions (Denbow, 2015).

The cecal microbial communities are more diverse in comparison to that of the ileum of chickens, and these different populations actively perform different functional roles (Pan & Yu, 2014). The primary functions of the cecum include fermentation of indigestible solubles to produce volatile fatty acids (VFA), converting uric acid to amino acids during reverse peristalsis, and absorption of amino acids, water, and electrolytes (Denbow, 2015; Mohd Shaufi et al., 2015; Krogdahl, 1985; Svihus et al., 2013). The functional roles of the bacterial communities within the ceca are emphasized by the morphology of the ceca itself. Through the transfer of uric acid into the ceca from the rectum via reverse peristalsis, digestion of bacterial cellular protein, and bacterial uric-acid-catabolism, gut bacteria and the cecum directly contribute to host nitrogen metabolism (Sergeant et al., 2014). The digestion of non-starch polysaccharides that takes place within the cecum has previously been assessed and the cecum was found to contain a high prevalence of species carrying genes coding for oligosaccharide degrading enzymes, specifically an abundance of sequences involved in the degradation of xylans (Sergeant et al., 2014). The cecal tubes found in the chicken GI tract are unique in functionality and their benefit to bird and microbial health.

The functions of these GI segments directly impact the composition of the microbial niche they harbor and vice versa (Denbow, 2015). The metabolic activities of specific indigenous bacteria in different sections of the bird's gastrointestinal (GI) tract contribute to proper GI function (Oakley et al, 2014). Ultimately, analyzing the gut microbiota's conspecific and heterospecific interactions and the biodiversity within gut biomes will provide information about the health of the animal and lead to a better understanding of enteric disease treatment and prevention (Mohd Shaufi et al., 2015).

3.2.1 Microbial Characterization Restrictions

Articulating a complete list of gut microbiota found in healthy broiler chickens using amplicon sequencing of the 16S rRNA gene has not yet been accomplished (Ballou et al., 2016). It is difficult to draw strong conclusions about the exact composition of the gut microbiota across different studies due to variability in primer selection, choice of GIT section, breed and age of the bird, housing conditions, diet, and other aspects of study design (Stanley et al., 2014; Clavijo & Flórez, 2018; Borda-Molina et al., 2018; Wei et al., 2013). In most cases, these variables elucidate the differences in reported microbiota compositions and must be considered when attempting to define a typical ileal or cecal microbial community (Borda-Molina et al., 2018). Because age is a significant determinant of microbial diversity, longitudinal studies offer a more complete depiction of the developing gut microbiota, though few have been conducted. Those that exist indicate that commercial broilers (Ross, Cobb, etc.) reach gut microbial stabilization between 14 and 21 days of age (Mohd Shaufi et al., 2015), after 20 days of age (Ijaz et al., 2018), or at least by 28 days (Ballou et al., 2016; Lu et al., 2003; Ocejo et al., 2019). As mentioned earlier, the choice of sequencing primer - the targeted hypervariable region(s) of the 16S gene - greatly affects the phylogenic profiling ability of the sequencing process. For these reasons, it

would be ideal that attempts at depicting the typical microbiota of the chicken ileum and cecum be based on results of studies conducted using birds from commercial broiler breeds of at least 28 days of age and primers targeting the V1-V4, V3, V3-V4, or V4 hypervariable regions. However, no universal standard - which would easily align study parameters - currently exists, muddying comparisons of results across available 16S rRNA amplicon sequencing studies (Borda-Molina et al., 2018). Nevertheless, 16S rRNA sequencing has been used successfully to create taxonomic profiles of the major genera and some minor bacterial genera of the chicken ileum and cecum by several researchers, despite possible variations in study design. It should be mentioned that 16S microbial profiling provides a much clearer estimate of the microbial members present than ever would have been possible with culture-dependent methods alone (Amit-Romach et al., 2004). Currently, some studies employ a metagenomic approach to assess the functionality of the present microbes, leading to an even more complete understanding of the gut microbiota (Yeoman et al., 2012).

3.2.2 Typical Ileal Microbiota

At present, there is no clear depiction of typical intestinal microbiota for healthy chickens, but many researchers have attempted to summarize the major and minor bacterial communities of the ileum and cecum (Kogut, 2019). Wei et al., 2013, conducted a bacterial census of the chicken intestinal microbiota using 16S rRNA sequences found in 3 public databases for nucleotide sequences: GenBank, Silva, and Ribosomal Database Project (RDP). Comparatively, a longitudinal study conducted by Mohd Shaufi et al., 2015, identified the major microbial members of the ileum and cecum at the genus level for different time points of the birds' development using 16S rRNA sequencing. Following suit, Xiao, et al., 2017, characterized the microbiota of the duodenum, jejunum, and ileum of 42-day-old broilers using 16S gene

sequencing. The mutual conclusions among these studies indicate that at the phyla level, the ileum is dominated by *Firmicutes, Bacteroidetes,* and *Proteobacteria*, even if the ratios are not identical (Wei et al., 2013; Mohd Shaufi et al., 2015; Xiao et al., 2017). Presently, Cuccato et al., 2021, has demonstrated that *Cyanobacteria* is an additional prevalent phylum of the chicken ileum. It has been reported that between 30 – 76% of the microbial population within the ileum of broilers is composed of *Lactobacillus* spp. of the phylum *Firmicutes*, demonstrating a substantial level of microbial homogeneity (Xiao et al., 2017; Lu et al., 2003; Clavijo & Flórez, 2018; Wang et al., 2016). Still, fluctuation in individual ileal abundance of *Lactobacillus* spp. over time and between studies is common (Mohd Shaufi et al, 2015). There is also much debate about which genus has the second greatest representation in the ileum. The minor genera of the ileal microbiota, those that are commonly detected at greater than 1% abundance but generally lower in abundance than *Lactobacillus* (Rychlik, 2020; Wei et al., 2013), typically include *Clostridium, Enterococcus,* (Latorre et al., 2018; Lu et al., 2003; Mohd Shaufi et al., 2015), *Bacteroides* (Xiao et al, 2017; Wei et al. 2013)*, Ruminococcus, Alistipes* (Wei et al., 2013*),* *Streptococcus* (Lu et al. 2003; Wang et al. 2016), and *Escherichia* (Wang et al., 2016) at varying reported abundances.

At the species level, *Lactobacillus salivarius* has been detected repeatedly and appears to be a ubiquitous resident of the ileum regardless of diet (Phong et al., 2010), or lumen or mucosal sample type (Gong et al., 2007). It is unclear whether this prevalent species is completely beneficial or harmful. Its role in the deconjugation of bile salt by *L. salivarius* and other lactobacilli potentially inhibits weight gain in broilers (Guban et al., 2006; Wang, Z. et al., 2012), yet in recent years, there is conflicting evidence that dietary supplementation of *L. salivarius* achieves the opposite effect, improving growth performance and promoting gut health

(Sureshkumar et al., 2021; Wang et al., 2020; Shokryazdan et al., 2017). Additionally, *L. salivarius* has been shown to inhibit the growth of the pathogenic *Salmonella enterica* (Zhou et al., 2007) and *E. coli,* (Wang et al., 2020). In general, *Lactobacilli* are considered beneficial bacteria, commonly used as probiotic treatments in *in vivo* trials (Phong et al., 2010; Sureshkumar et al., 2021; Nakphaichit et al., 2011; Wang, J., et al., 2020). In addition to *L. salivarius, Lactobacillus aviaries* (Gong et al., 2007)*, Enterococcus cecorum* (Bjerrum et al., 2006)*, Lactobacillus crispatus* (Wang et al., 2016)*, Lactobacillus reuteri* (Wang et al., 2016; Lu et al., 2008), and *Lactobacillus acidophilus* (Lu et al, 2008; Lu et al., 2003) are frequently identified bacterial species; stabilizers of the ileal microbiome.

The digestive functions of the gastrointestinal tract take place under specific environmental conditions. Although the ileum generally has a neutral pH (6.3-6.7) (Denbow, 2015), bacteria must be able to withstand the low pH in the crop, proventriculus, and gizzard before arriving in the ileum. Because of this, the ileum selectively permits the survival of microbes that can withstand and benefit from the conditions of the environment inside the GIT, especially lactic acid bacteria (LAB) which exhibit strong resistance to bile salt and low pH (Bukhari et al., 2017). In addition to a low pH and bile salt tolerance, dominant ileal microbes are those that competitively adhere to ileal epithelial cells, exhibit cell surface hydrophobicity, and a resistance to high phenol concentrations (Reuben et al., 2019). This understanding is in corroboration with the previously reported phylogenetic findings listed above, describing LAB as the dominant members of the ileal microbiota. So, while there is not a definitive ileal microbial directory, the archetypal microbes in a commercial broiler small intestine should display the properties that permit survival under the environmental conditions within the host.

3.2.3 Typical Cecal Microbiota

More work has been done to describe the cecal microbiome, as it is typically more diverse and hosts a greater concentration of microbes than the ileum, yet the same restrictions regarding study design variability and its effect on ileal microbiome characterization persist. In the ceca, taxonomic richness and diversity typically increase starting on the day of hatch and foster a thriving microbial community by market age (six weeks of age) (Oakley, et al., 2014). The key members of the chicken microbiota at first hatch are almost fully replaced by other bacteria by market age. This age-related shift usually involves the replacement of most of the aerobic, Gram (-) species with anaerobic, Gram (+) species (Ballou et al., 2016). It is uncontested that the typical broiler cecum is dominated at this time by *Firmicutes* and *Bacteroidetes* followed by *Proteobacteria* and *Actinobacteria* at the phyla level (Rychlik, 2020; Oakley et al., 2014; Clavijo & Flórez, 2018; Mancabelli et al., 2016; Lan et al., 2002). As in the case of the ileum, there is wide variability in the makeup of the cecal microbiota, but a few genera and species are commonly sequenced and/or found to be abundant in the cecum across multiple studies. Early sequencing efforts indicated that the known genera *Clostridium, Eubacterium,* and *Ruminococcus* generally represent the bulk of total cloned sequences found in the chicken cecum (Zhu et al., 2002; Lu et al., 2003; Bjerrum et al., 2006; Wei et al., 2013), but current work points to *Bacteroides* and *Faecalibacterium,* in addition to *Ruminococcus, Clostridium,* and *Eubacterium* as primary genera (Cuccato et al., 2021; Glendinning et al., 2019; Ocejo et al., 2019; Mohd Shaufi et al., 2015). There is conflict among these studies in ranking the abundances of these genera and a unanimous conclusion has not been made. The heightened bacterial density and diversity within individual cecal biomes, and even between healthy birds, makes it difficult to label any genus, or especially species, as definitively dominant within the cecum. Rather,

observable trends in microbial populations highlight the common members. In this aspect, *Faecalibacterium prausnitzii, Escherichia coli, Escherichia shigella,* and *Clostridium* cluster IV and XIV*,* and strains related to these species, appear to be of interest within the cecum of healthy broilers (Mohd Shaufi et al., 2015; Awad et al., 2016; Ijaz et al., 2018; Zhu et al., 2002; Bjerrum et al., 2006; Amit-Romach et al., 2004; Gong et al., 2007). *F. prausnitzii,* and the *Clostridium* clusters IV and XIV are usually described as commensal gut bacteria that contribute to butyrate production within the cecum, aiding in epithelial barrier function (Bjerrum et al., 2006; Rinttilä & Apajalahti, 2013; De Maesschalck et al., 2015). In contrast, pathogenic *E. coli and E. shigella* are common residents of the broiler cecum*,* though their numbers decline as the birds age (Seidavi et al., 2010). The ability to suppress pathogen growth in the gut is directly related to the composition of the adult cecal microbiota (Baba et al., 1991). In the case of enteric diseases, there is an expectation that negative microbial shifts that take place either trigger or are a response to disease challenges.

3.3 Microbiota Modulation during Necrotic Enteritis Challenge

The chicken intestinal microbiota community structure typically shifts during NE outbreak to a state known as dysbiosis. This state refers to an overgrowth of pathogenic microbes causing a balance shift within the microbial community that is typically not found in a healthy bird. This type of shift is associated with environmental changes including pH instability, increased mucous production, and reduced intestinal transit time of digesta (Latorre et al., 2018). The microbial communities within the ileum and cecum are commonly examined for fluctuations during a NE challenge. Both locations bear a distinct microbial community and significant changes in the composition of those communities can lead to different physiological responses by the host. When characterizing modulations of the gut microbiota, each shift in the microbial

composition is evaluated extensively. Anything from the environment, management style, diet, use of feed additives, antibiotics, vaccines, to the breed and age of the bird all affect the intestinal microbiota (Mohd Shaufi, et al., 2015).

3.3.1 Role of Clostridium perfringens during Necrotic Enteritis Dysbiosis

C. perfringens is naturally found within the digestive tracts of chickens and mammals, but there are several previously discussed predisposing conditions that can lead to an increase in the abundance of *C. perfringens.* Under normal conditions, *C. perfringens* can be found in the GIT of healthy chickens, but the population increases to an alarming density during NE infection (Fasina et al., 2016). The cecum hosts a higher concentration of *C. perfringens* in healthy birds, but necrotic lesions in infected birds are more obvious in the small intestine (Stanley et al., 2012). The cecum of birds predisposed to NE and inoculated with *C. perfringens* has been found to harbor about $10^{6.9}$ CFU/g digesta, while the ileum contained roughly $10^{6.1}$ CFU/g digesta (Craven, 2000). Though not typically considered a beneficial bacterium under normal conditions, with limited accessibility to colonization, it is also not usually considered a direct threat to animal health.

In cases where predisposition to NE occurs, as mentioned previously, *C. perfringens* can quickly damage the intestinal tract with a specialized approach. Gross intestinal lesions across the small intestine and cecum, a thin, fragile intestinal wall, and flock mortality of at least 1% are typical diagnostic factors indicative of NE (Olkowski et al., 2006; Helmboldt & Bryant, 1971). To accomplish this, *C. perfringens* uses its aggressive collection of virulence factors, including more than 20 known toxins. These toxins inhibit the host immune response by blocking the differentiation of neutrophils, weakening the intestinal barrier function (Takehara et al., 2016). Until recently, *C. perfringens* Type A and C alpha toxin, as well as Type C beta-toxin, were

thought to be the primary causative agents of NE. The result of more current work disproves this previous impression, and it is widely accepted that a recently discovered NetB toxin is likely more responsible for the pathogenesis of NE (Latorre et al., 2018). It is not entirely understood how the NetB toxin contributes to NE because the specific receptor has not yet been identified, but it is known that this is a pore-forming toxin, which ultimately causes cell lysis (Zaragoza et al., 2019).

C. perfringens rely on more than toxin production to infect and damage a host. Recently, genes coding for antimicrobial resistance and collagen adhesion have been identified, enhancing the detrimental potency to a bacterium already capable of toxin production (Kiu et al., 2019). The gene *cnaA* is the initial gene in a five-gene sequence collectively referred to as the collagen adhesin (CA) locus, a polycistronic operon (Wade et al., 2016). The prevalence of the adhesin-encoding gene *cnaA* in NE diseased birds is specifically important because it has also been demonstrated that *C. perfringens* ability to cause NE is strongly positively correlated with its ability to bind to collagen, aiding the bacteria in the colonization of the GI tract (Wade et al., 2016). The ability to adhere to collagen type IV, the dominant collagen type found in the basement membrane of the intestines, was inhibited in mutations of the EHE-NE18 strain of *C. perfringens*, in which the *cnaA* gene was inactivated (Wade et al., 2016).

Under normal conditions, healthy broilers are virtually immune to the effects of *C. perfringens.* When other diseases like coccidiosis are introduced or when birds are fed an inadequate diet or are experiencing elevated levels of stress due to overcrowding, NE becomes a major concern. It is then that the ability of *C. perfringens* to rapidly proliferate and expel its armory of toxins and evasion techniques are threatening to the health of the flock. These predisposing conditions –

alone or in combination with exposure to elevated levels of *C. perfringens* – induce dysbiosis, with some bacteria being affected similarly across several studies.

3.3.2 Microbial Modulation by Predisposition

Typically, increasing *C. perfringens* alone will not alter the microbial balance significantly, so most reports include the microbial modulation as related to different predisposing factors plus exposure to *C. perfringens.* Predisposition involving fishmeal diets and *Eimeria* exposure have been known to affect the microbial composition of the diseased state differently. Wu, et al., 2014, found that a fishmeal diet has a greater effect on the cecal microbiota than a coccidia challenge alone, but when combined the greatest number of OTU abundances are affected. Interestingly, Stanley et al., 2014, found that *Eimeria,* alone or combined with *C. perfringens* challenge, had a greater effect on microbial composition than a fishmeal diet. What is clear is that the combination of a fishmeal-based (high protein) diet, and *Eimeria* and *C. perfringens* challenges significantly alter the microbiota composition of the ceca.

The microbial community of the cecum is affected by different predisposing factors to NE, both with and without *C. perfringens* challenge and, specifically, the depletion of *Lactobacillus johnsonii* is common (Antonissen et al., 2016; Stanley et al., 2014; Wu et al., 2014). Less commonly, the abundance of cecal *Lactobacillus* increases during the *C. perfringens* challenge (Gharib-Naseri et. al., 2019). In the case of Macdonald, et al., 2017, an increase of *L. johnsii* was observed in response to asymptomatic infection solely induced by *Eimeria*. When more severe cecal lesions were induced, a significant decrease in *Lactobacillus reuteri* and *L. pontis* was observed (Macdonald et al., 2017). Similarly, there is conflicting evidence about the abundance of *Ruminococcus* spp. in differentially infected chickens. In the ileum of chickens challenged with *Eimeria*, fishmeal, and *C. perfringens,* an increase of *Ruminococcus* spp. was observed. (Xu

et al., 2018), but when birds were challenged with *Eimeria* and *C. perfringens* only, a reduction was observed (Bortoluzzi et al, 2019). In contrast, Latorre et al., 2018, reported an increase in ileal *Ruminococcus* in response to *Eimeria, C. perfringens,* and *Salmonella* Typhimurium. In the cecum, the reduction of *Ruminococcus* spp. in variably challenged birds is more commonly observed, with less opposition (Stanley et al., 2012; Wu et al., 2014; Bortoluzzi et al., 2019; Gharib-Neseri et al., 2019). Again, there is not yet a standard expectation for microbiota modulation during NE, just as there is not a definitive list of intestinal microbiotas typical of a healthy broiler. Following this philosophy, a chosen treatment for NE should not target the reduction of any specific microbiota, but rather, provide aid to the already existing characteristics and functions of the chicken GIT.

4. Explored Methods for the Prevention and Treatment of Necrotic Enteritis

Because NE is the result of compounding predisposing factors rather than a single-sourced disease, mitigation of NE through bird and house management is promising. As discussed previously, the major predisposing factors to NE are coccidiosis, improper diet, and housing conditions, and concurrent pathogen-induced dysbiosis. These factors cause stress on the animal and weaken the immune system of young chicks which increases the risk of NE infection (Tsiouris, 2016). The proliferation of *C. perfringens,* once instigated by these predisposing factors, is exacerbated by its array of toxins, including α-toxin, NetB, TpeL, and potentially, others that have yet to be identified (Alizadeh, et al., 2021). It is these toxins that are responsible for the disease NE. Mitigating toxin production during NE is essential to disease prevention and animal recovery. Finally, proposed treatments for NE outbreaks include butyrate and pre-and probiotic supplementation.

4.1 Preventing Coccidiosis

Coccidiosis is caused by the protozoa *Eimeria,* commonly, *E. maxima, E. acervulina, and E. tenella.* An anticoccidial drug, toltrazuril, has been found to reduce necrotic lesions caused by coccidiosis in young birds that lead to NE, but this drug should preferentially be administered after the onset of coccidiosis has begun to reduce drug resistance (Alnassan et al., 2013). The advent of coccidia vaccination offers a prophylactic approach. Anticoccidial vaccines containing several strains of *Eimeria* spp. alleviate the risk of drug resistance during a live-attenuated vaccine trial and offer an easy administration procedure via deep litter spray (Bangoura B., et al., 2014). There is some discrepancy in bird performance after vaccine administration. Some studies have shown that anticoccidial vaccines led to low body weight and increased feed conversion ratio for young birds, but other studies reported compensatory weight gain and comparable bird weight at the end of grow-out (Lee et al., 2011). The negative effects of coccidia vaccines on bird performance are not ideal, but they appear to level out as the birds age (Cowieson et al., 2020). Nevertheless, because of the reported losses in animal performance related to vaccine administration, the control of coccidiosis is being further explored. New data supports the use of phytochemicals including the thyme and oregano-derived compounds thymol and carvacrol, respectively, which reduce inflammation, possess antimicrobial properties against enteric pathogens, including Clostridia spp., and stimulate enteric enterocyte production, strengthening the mucosal immune barrier function (Broom, 2017; Gholami-Ahangaran et al., 2020). With this method, there is concern over potential toxicity, as with any compound, and more work is needed to determine a safe dosage of these phytochemicals. These various methods of preventing and controlling coccidiosis are currently being studied to rule out the most effective approach.

4.2 Prevention through Diet

Poultry diets known to predispose birds to NE are those that include high protein levels, animal-derived protein, and cereal grains including rye, oats, wheat, and barley. These cereal grains are problematic due to the high non-starch polysaccharide, or complex carbohydrate content which increases digesta viscosity and decreases intestinal transit time which aids in *C. perfringens* overgrowth (Dahiya et al., 2006). Interestingly, cereal grains that are less processed are beneficial to gizzard function, in turn reducing *C. perfringens* proliferation. When whole wheat is added as a portion of the diet, decreased intestinal counts of *C. perfringens* and gizzard pH have been reported (Dahiya et al., 2006). Feeds containing high levels of animal-derived protein sources, primarily fishmeal, are advantageous for *C. perfringens* proliferation due to the excess nutrient supply and pH alteration in the GIT (Moore, 2016). When comparing the occurrence of NE in birds fed either a fishmeal or soy protein-based diet, it was found that certain amino acids may contribute to the increase in NE. Dietary levels of glycine and to a lesser extent, methionine, are higher in fishmeal diets compared to soy, and a positive correlation between glycine concentration and *C. perfringens* colonization in the ileum and cecum has been reported (Dahiya et al., 2006). More work is still needed to fully understand the relationship between amino acid concentration and NE, but overall removing animal-based protein from the broiler diet is effective for managing NE. Instead, feeding corn- and/or soy-based diets that meet the National Research Council (NRC, 1994) guidelines for protein concentration are an excellent alternative.

4.3 Prevention through Animal Husbandry

Necrotic enteritis is spread via the fecal-oral route making house management crucial to the prevention of this disease. Because birds are housed in close proximity to one another and share bedding, feeders, and waterers, the potential for rapid spread skyrockets. The number of birds or

the total live weight of birds (kg) in a broiler house at the same time per square meter of the usable area is referred to as stocking density (Tsiouris et al., 2015b). In general, as stocking density increases, animal performance, welfare, and bird health decline (Tsiouris, 2016). In one study where "high stocking density" was represented by 30 birds per square meter and the control or "normal stocking density" was represented by 15 birds per square meter, high stocking density was reported to significantly increase the occurrence and severity of NE as well as counts of *C. perfringens* in the ceca (Tsiouris et al., 2015b). According to the National Chicken Council, whose mission is to influence legislation involving poultry production in the United States, the recommended stocking density for broilers between 5.6 and 7.5 pounds at live weight is 8.5 pounds live weight per square foot of usable space (National Chicken Council, 2020). The average live weight at the end of grow-out in the U.S. was 6.46 pounds per bird in 2021 (National Chicken Council, 2022). The stocking density recommended by the National Chicken Council equates to roughly 14 birds per square meter using an average live weight of 6.5 pounds and a stocking density of 8.5 pounds live weight per square foot of usable space. Using this recommendation for stocking density is one way to avoid the stress associated with overcrowding that predisposes birds to NE. Additionally, adequate feeder and watering space per bird, house temperature and humidity, lighting procedures, and litter conditions affect bird health and immune response and should be major considerations for producers when designing a management strategy (Moore, 2016; Tsiouris, 2016).

4.4 Prevention through Vaccination

Diseases threatening human and animal health that arise from pathogens in the *Clostridium* genus are common. Tetanus, botulism, blackleg, gas gangrene, and NE affect animals throughout livestock production for animal consumption. Famously, humans receive regular tetanus "shots"

containing tetanus toxoid to prevent this gruesome disease. These common toxoid vaccines are created by heating or adding formaldehyde to purified bacteria exotoxins, e.g., the tetanus neurotoxin (Yadav & Khurana, 2020). The injection of this toxoid stimulates an immune response in the host to the inactive toxoid and provides lasting protection, with a recommended booster vaccine every 10 years (Hall et al., 2021). Tetanus was once analogous to a death sentence for humans, but with the use of toxoid vaccines in the last century, the United States has almost completely halted reported cases and successfully reported no deaths associated with tetanus in 2018 (Hall et al., 2021).

Like *Clostridium tetani, C. perfringens* produce an array of strain-specific toxins which lead to several different diseases, but no single strain can produce all the known toxins concurrently (Zaragoza et al., 2019). These *C. perfringens* strains have been classified into seven toxinotypes (A-G) based on the toxin(s) they produce: alpha (CPA), beta (CPB), epsilon (ITX), *C. perfringens* enterotoxin (CPE), NE beta-like (NetB) (Zaragoza et al., 2019). It was historically thought that the alpha toxin produced by all toxinotypes was the primary virulence factor of NE in chickens, but when a CPA deficient mutant strain triggered NE in a challenge setting this was disproven (Keyburn et al., 2008). This study suggested that a novel (NetB) toxin produced by type G strain *C. perfringens* is the main antagonist to NE (Keyburn et al., 2008). However, there is a strong correlation between the occurrence of NE in broilers and the presence of the type A and C strains, which had previously led researchers to believe CPA played a role in the pathogenesis of NE (Lovland et al., 2004; Cooper et al., 2009; Zaragoza et al., 2019).

The development of toxoid vaccines for NE prevention in broiler chickens has been studied extensively. Vaccination of hens using the type A (CPA) and C (CPA, CPB) toxoids provided partial protection to broiler chicks through maternal antibody transmission in the egg yolk, with

the type C toxoid outperforming the type A (Lovland et al., 2004). In broilers, however, maternal vaccination provides short-lived antibody protection in progeny which is less helpful to young birds at 3-4 weeks of age, when NE typically occurs (Mot et al., 2014). Cooper, et al., 2009 confirmed that the antibody response to a recombinant CPA toxoid partially protects birds from experimental NE, but the role of the alpha-toxin in pathogenesis was not entirely understood. Some of the first work investigating the potential for a NetB toxoid vaccine concluded that a recombinant NetB (rNetB) toxin could effectively protect broiler chicks from mild NE and that the best protection came from birds immunized with cell-free toxoid or bacterin supplemented with rNetB (Keyburn et al., 2008). This work also revealed that rNetB toxoid alone was insufficient at protecting birds against a more severe challenge. Because the CPA and NetB toxoid vaccines had been shown to independently provide partial protection against NE challenge, an investigation into the effect of a NetB and CPA combination vaccine took place. Again, this combination yielded only complete protection in a subclinical, mild challenge and only partial protection was observed during a more severe challenge (Fernandes da Costa et al., 2016). Additionally, the subcutaneous administration of the vaccine would not be feasible in a commercial setting, but more work will need to be conducted to determine a more suitable vector (Fernandes da Costa et al., 2016). In summary, vaccine development for NE in chickens is challenging due to the complexity of the toxins produced by *C. perfringens* and because a vector of administration suitable for a commercial broiler setting has not yet been tested. A more detailed evaluation of the challenges associated with vaccine development is laid out by Mot, D., et al., 2016, but this discussion is beyond the scope of this review.

4.5 Treatment through Butyrate Supplementation

Without access to antibiotics to combat NE, researchers are currently investigating the antimicrobial potential of organic acids, including butyric acid-derived compounds. Butyrate, the short-chain fatty acid (SCFA) known as butyric acid, is naturally produced through microbial fermentation in the GIT of many species and performs several roles in intestinal regulation. Primarily, butyrate enhances epithelial cell proliferation which supports intestinal barrier function. The intestinal barrier is created and maintained by tight junctions of epithelial cells whose function is to prevent leakage of pathogens and non-soluble nutrients outside of the intestinal lumen (Baumgart & Dignass, 2002). Sodium butyrate, a butyrate salt, has been shown to promote the expression of the tight-junction protein Claudin-1, which is vital to intestinal barrier permeability regulation (Wang, H. B. et al., 2012). It has been established that butyric acid promotes intestinal epithelial cell growth and relieves irritable bowel disease in humans by strengthening the intestinal barrier (Plöger et al., 2012). Butyrate also serves as a nutrient source for epithelial cells (Bedford & Gong, 2018), further aiding epithelial cell growth and function. Additionally, butyrate is known to reduce inflammation in the intestinal epithelium. This is likely the result of reduced pro-inflammatory cytokine expression (IFN-y, TNF-a, IL-1B, IL-16, IL-8) and increased expression and signaling of anti-inflammatory cytokines IL-10 and TGF-B, (Bedford & Gong, 2018). This points to the interaction between butyrate supplementation and the inhibition of the inflammatory pathway NF-kB for alleviating symptoms of human enteric diseases (Bedford & Gong, 2018). Because chickens are commonly used as a model for human health in research, it is likely that butyrate functions similarly in chickens.

There are also reported butyrate-induced improvements in animal performance that are important to the production of broilers for human consumption and favor its profitability. The performance

parameters of average daily gain (ADG), feed efficiency, and quality parameters including fat deposition and serum cholesterol have been studied in relation to butyrate supplementation. When broiler chickens were fed butyrate derivatives an increase in blood glucose levels, a decrease in percent fat deposition in breast muscle, and a reduction in serum cholesterol levels were observed, however, no significant differences in growth performance were observed (Bedford et al., 2017). In pigs, however, the potential of butyrate derivative supplementation to enhance growth performance has been established. The butyrate glyceride, tributyrin, improved ADG and decreased fecal scores in weaning-stressed piglets, a time when piglets are prone to intestinal tract disorders (Wang, C. et al., 2019). The beneficial effect of butyrate supplementation is further demonstrated by the results of experiments using healthy, weaned piglets. For instance, Sotira, 2020, found that 0.5% tributyrin in the diet improved feed efficiency, increased weight gain and serum glucose while decreasing serum urea, an indicator of lean tissue growth. While the performance benefits of butyrate feed additives are obvious in pigs, it is possible that too few experiments involving butyrate and butyrate glyceride supplementation on broiler chickens have been performed to fully determine the effect on their growth performance.

4.5.1 Challenges of Using Butyrate-Derived Compounds

Butyrate supplementation is excellent for reducing inflammation, strengthening the intestinal barrier function, and serving as an energy source for luminal epithelial cells. Although free butyrate is the most effective form of butyric acid in-vitro, in this form it is too quickly absorbed, corrosive, and unpalatable to use directly in feed for in-vivo experimentation (Li et al., 2015). Encapsulating butyric acid in a lipid matrix increases absorption time, dispersing butyric acid into the proximal small intestine, where contact with *C. perfringens* is more likely and butyrate

function is more effective (Hofacre et al., 2020; Bedford & Gong, 2018). These encapsulated butyrate salts, such as sodium butyrate, have commonly been used to study the effect of butyrate supplementation on the chicken gut, but this method is costly, time-consuming, and most importantly, decreases the butyrate concentration (Lum et al., 2018). To combat the challenges of using encapsulated butyrate, butyrate has been anchored to glycerides to form derivatives like mono-, di-, and tributyrin, which are being explored further (Li et al., 2015). Tributyrin is a triacylglycerol ester of butyrate which does not require the same encapsulation process to deliver butyrate to the small intestine and is at least equally as effective at improving feed conversion rate and body weight gain as sodium butyrate (Lum et al., 2018). Similarly, sodium butyrate, when compared to butyrate glycerides, had similar effects on intestinal morphology development (Bedford & Gong, 2018).

4.5.2 Butyrate Antimicrobial Effects

In addition to the known benefits of butyrate on intestinal health regulation, there is some existing evidence that butyrate has potential antimicrobial effects. Butyrate supplementation has repeatedly inhibited *Salmonella* growth in *in vitro* and *in vivo* studies targeting the chicken ceca. In an *in vitro* trial, *Salmonella* Enteritidis growth was inhibited by the short-chain fatty acids, propionate, acetate, and butyrate at a pH of 6.0, with a greater emphasis on the effects of propionate and butyrate, the concentration of the SCFA, and pH level (Van Immerseel et al., 2003). When delivered in a wax matrix, sodium butyrate inhibited *Salmonella* Enteritidis in the cecum of broilers (Onrust et al., 2020). Namkung, et al, 2011, found that *Salmonella* Typhimurium and *Clostridium perfringens* were best inhibited by *n*-butyric acid, or free butyric acid, compared to butyrate derivatives, however *C. perfringens* required a higher concentration of *n*-butyrate than *Salmonella*. The mechanisms by which butyrate modulates the intestinal

microbiota are not well understood, but it is believed that the pathogen control observed by butyrate supplementation is an indirect effect of butyrate's interaction with the host (Riaz, 2017; Guilloteau et al., 2010). Additionally, the pH level, butyrate concentration, mode of delivery, release rate, and location, all play a role in the effectiveness of butyrate as an antimicrobial feed additive (Guilloteau et al., 2010; Onrust et al., 2020; Namkung et al., 2011; Banasiewicz et al., 2020).

4.6 Treatment through Pre- and Probiotic Supplementation

Pre- and probiotics have gained much attention as potential replacements for antibiotics in poultry and livestock production. Prebiotics are indigestible feed ingredients, commonly non-starch polysaccharides, which provide a food source to - and stimulate the growth of - beneficial gut bacteria, without directly feeding any microbial to the animal (Dahiya et al., 2006; Mora et al., 2020). The International Scientific Association for Probiotics and Prebiotics defines a prebiotic as "a substrate that is selectively utilized by host microorganisms conferring a health benefit" (Froebel et al., 2019). Orally administering prebiotics has been shown to decrease the intestinal population of *C. perfringens* in chickens, as well as promote the growth of lactic acid bacteria (LAB) which outcompete pathogenic bacteria in the GIT (Froebel et al., 2019). Probiotics, or direct-fed-microbials (DFM), are defined as "live microbial feed supplement which beneficially affects the host animal by improving its intestinal balance" (Dahiya et al., 2006; Fuller, 1989). The intestinal tract of newly hatched chicks is sterile, and colonization of gut microbiota from the local environment takes place over time, experiencing shifts in bacterial dominance (Dahiya et al., 2006). The chicken GIT microbial community becomes stable at an unconfirmed time point, with fluctuations halting at 3, 14, 21, 25, or 28 days after hatch, in various literature (Mohd Shaufi et al., 2015; Awad et al., 2016). Between day-of-hatch and

approximately 3-4 weeks of age, birds are most susceptible to NE infection (Zahoor et al., 2018). Oral supplementation of beneficial bacteria to young birds could quick-start the colonization of normal gut microbiota found in healthy adult birds, and potentially prevent NE through the competitive exclusion of pathogens (Dahiya et al., 2006). Additionally, a mixture of prebiotics and probiotics referred to as synbiotics provides the GIT with not only the DFM but with an immediately available substrate, improving the survival of the DFM (Mora et al., 2020). Few studies have been conducted evaluating their use in treating NE in chickens (Mora et al., 2020).

4.6.1 Prebiotics for the Treatment of Necrotic Enteritis

The ideology of using prebiotics in poultry production stems from their ability to enhance the morphology within the GIT, as well as improve feed efficiency and growth performance through the production of SCFA (including butyrate) by fermentation of the prebiotic substrate (Mora et al., 2020). There is little available information about the use of prebiotics as a treatment for NE in broilers, and most work studying the effect of prebiotics on *C. perfringens* was conducted on mammals and *in vitro* techniques (Dahiya et al., 2006). Reduction in the intestinal population of *C. perfringens* has been observed in chickens fed a dextrose-isolated soy protein diet containing 4 g/kg of short-chain fructooligosaccharides (SCFOS) or mannan-oligosaccharides (MOS) when compared to birds fed a corn-based diet without prebiotic supplementation (Biggs et al., 2007). The birds in this study were not undergoing a NE challenge, however. More recently, research investigating the effect of MOS with yeast culture on growth performance and pathogen exclusion concluded that this prebiotic did not significantly reduce counts of *C. perfringens* in the ileum or cecum or have a significant effect on total LAB (Froebel et al., 2019). There is debate on whether MOS can be considered a prebiotic by definition because they have not been shown to alter intestinal microbiota, although they do enhance animal performance in several

animal species (Ducatelle et al., 2015). The role of prebiotics in treating NE in broilers may be overshadowed by the more heavily studied use of probiotics for the same goal.

4.6.2 Probiotics for the Treatment of Necrotic Enteritis

More research has been conducted to explore the efficacy of the treatment of NE with probiotics than with prebiotics. Lactic acid bacteria (LAB), including *Lactobacillus,* and *Bifidobacterium* genera, are frequently used as probiotics in humans and animals (Ducatelle et al., 2015). Other LAB genera that may help treat NE in chickens include *Bacillus, Enterococcus,* and *Saccharomyces* (Dahiya et al., 2006; Mora et al., 2020). There is evidence that the LAB probiotic containing *Lactobacillus salivarius and Pediococcus parvulus* decreases the severity of NE infection and colony counts of *C. perfringens* in the chicken small intestine (Layton et al., 2013). *Lactobacillus salivarus* has also been shown to improve body weight gain, feed conversion ratio, immune response, intestinal morphology, and increase SCFA production in the small intestine, making it a promising candidate for the probiotic treatment of NE and enhanced bird performance (Wang, J. et al., 2020; Sureshkumar et al., 2021). Another LAB, *Bacillus licheniformis,* has been shown to prevent dysbiosis in the ileum microbial community in birds challenged with *C. perfringens* and *Eimeria* (Xu et al., 2018). The birds challenged with NE and orally administered *B. licheniformis* in this study exhibited ileum microbiota communities similar to the negative control group, whereas the challenged birds exhibited increased *Bacteroides* and *Ruminococcus* spp., which can be harmful to the host when the natural population is over-represented in the GIT (Xu et al., 2018). An additional LAB, E*nterococcus faecium,* when fed to birds with experimentally induced NE, protected the intestinal barrier from severe lesions associated with NE, possibly by modulating cytokine expression and intestinal *Lactobacillus* populations (Wu et al., 2019). In conclusion, several LAB have the potential to

replace antibiotics as a treatment for NE in broiler chickens, with different advantages and mechanisms of action. Importantly, probiotics in animal production do not appear to pose a threat to human health or medicine in the way that antibiotics did, making probiotics a favorable choice for NE treatment.

5. Summary of Literature Review

The removal of antibiotics from poultry production has highlighted an area in disease control that needs improvement. The pathogenesis and treatment of NE in chickens, as well as the relationship this disease has with the intestinal microbiota, has historically been difficult to understand, and currently, there is still work to be done. By using the most current technology for bacterial classification to examine the potential methods of treatment and prevention in relation to the chicken intestinal microbiota, we are closer to finding a treatment for NE than ever. Equally, by improving the bird environment and management we can take steps to effectively prevent and control this disease in the future.

LITERATURE CITED

Alizadeh, M., Shojadoost, B., Boodhoo, N., Astill, J., Taha-Abdelaziz, K., Hodgins, D. C., ... & Sharif, S. (2021). Necrotic enteritis in chickens: a review of pathogenesis, immune responses and prevention, focusing on probiotics and vaccination. Animal Health Research Reviews, 22(2), 147-162.

Allali, I., Arnold, J. W., Roach, J., Cadenas, M. B., Butz, N., Hassan, H. M., ... & Azcarate-Peril, M. A. (2017). A comparison of sequencing platforms and bioinformatics pipelines for compositional analysis of the gut microbiome. BMC microbiology, 17(1), 1-16.

Alnassan, A. A., Shehata, A. A., Kotsch, M., Schrödl, W., Krüger, M., Daugschies, A., & Bangoura, B. (2013). Efficacy of early treatment with toltrazuril in prevention of coccidiosis and necrotic enteritis in chickens. Avian Pathology, 42(5), 482-490.

Amit-Romach, E., Sklan, D., & Uni, Z. (2004). Microflora ecology of the chicken intestine using 16S ribosomal DNA primers. Poultry science, 83(7), 1093-1098.

Annett, C. B., Viste, J. R., Chirino-Trejo, M., Classen, H. L., Middleton, D. M., & Simko, E. (2002). Necrotic enteritis: effect of barley, wheat and corn diets on proliferation of Clostridium perfringens type A. Avian Pathology, 31(6), 598-601.

Antonissen, G., Eeckhaut, V., Driessche, K. V., Onrust, L., Haesebrouck, F., Ducatelle, R., … Immerseel, F. V. (2016). Microbial shifts associated with necrotic enteritis. Avian Pathology, 45(3), 308–312. doi: 10.1080/03079457.2016.1152625

Awad, W. A., Mann, E., Dzieciol, M., Hess, C., Schmitz-Esser, S., Wagner, M., & Hess, M. (2016). Age-related differences in the luminal and mucosa-associated gut microbiome of broiler chickens and shifts associated with Campylobacter jejuni infection. Frontiers in cellular and infection microbiology, 6, 154.

Baba, E., Nagaishi, S., Fukata, T., & Arakawa, A. (1991). The role of intestinal microflora on the prevention of Salmonella colonization in gnotobiotic chickens. Poultry science, 70(9), 1902-1907.

Ballou, A. L., Ali, R. A., Mendoza, M. A., Ellis, J. C., Hassan, H. M., Croom, W. J., & Koci, M. D. (2016). Development of the chick microbiome: how early exposure influences future microbial diversity. Frontiers in veterinary science, 3, 2.

Banasiewicz, T., Domagalska, D., Borycka-Kiciak, K., & Rydzewska, G. (2020). Determination of butyric acid dosage based on clinical and experimental studies–a literature review. Przeglad gastroenterologiczny, 15(2), 119.

Bangoura, B., Alnassan, A. A., Lendner, M., Shehata, A. A., Krüger, M., & Daugschies, A. (2014). Efficacy of an anticoccidial live vaccine in prevention of necrotic enteritis in chickens. Experimental parasitology, 145, 125-134.

Baumgart, D. C., & Dignass, A. U. (2002). Intestinal barrier function. Current Opinion in Clinical Nutrition & Metabolic Care, 5(6), 685-694.

Bedford, A., & Gong, J. (2018). Implications of butyrate and its derivatives for gut health and animal production. Animal Nutrition, 4(2), 151-159.

Bedford, A., Yu, H., Squires, E. J., Leeson, S., & Gong, J. (2017a). Effects of supplementation level and feeding schedule of butyrate glycerides on the growth performance and carcass composition of broiler chickens. Poultry Science, 96(9), 3221-3228.

Biggs, P., Parsons, C. M., & Fahey, A. G. (2007). The effects of several oligosaccharides on growth performance, nutrient digestibilities, and cecal microbial populations in young chicks. Poultry science, 86(11), 2327-2336.

Bjerrum, L., Engberg, R. M., Leser, T. D., Jensen, B. B., Finster, K., & Pedersen, K. (2006). Microbial community composition of the ileum and cecum of broiler chickens as revealed by molecular and culture-based techniques. Poultry science, 85(7), 1151-1164.

Borda-Molina, D., Seifert, J., & Camarinha-Silva, A. (2018). Current perspectives of the chicken gastrointestinal tract and its microbiome. Computational and structural biotechnology journal, 16, 131-139.

Bortoluzzi, C., Vieira, B. S., Hofacre, C., & Applegate, T. J. (2019). Effect of different challenge models to induce necrotic enteritis on the growth performance and intestinal microbiota of broiler chickens. Poultry science, 98(7), 2800-2812.

Broom, L. J. (2017). Necrotic enteritis; current knowledge and diet-related mitigation. World's Poultry Science Journal, 73(2), 281-292.

Bukhari, S. M., Iram, M., Lijie, T., Sunting, M., Mang, H. L., Abbas, G., ... & Li, Y. (2017). Coherence and colonization characteristics of recombinant Lactobacillus under simulated gastric conditions within chicken GI tract and its impact on chicken growth. Pak. Vet. J, 37, 381-386.

Chakravorty, S., Helb, D., Burday, M., Connell, N., & Alland, D. (2007). A detailed analysis of 16S ribosomal RNA gene segments for the diagnosis of pathogenic bacteria. Journal of microbiological methods, 69(2), 330-339.

Chapman, H. D., Jeffers, T. K., & Williams, R. B. (2010). Forty years of monensin for the control of coccidiosis in poultry. Poultry science, 89(9), 1788-1801.

Clavijo, V., & Flórez, M. J. V. (2018). The gastrointestinal microbiome and its association with the control of pathogens in broiler chicken production: a review. Poultry science, 97(3), 1006-1021.

Collier, C., Hofacre, C., Payne, A., Anderson, D., Kaiser, P., Mackie, R., & Gaskins, H. (2008). Coccidia-induced mucogenesis promotes the onset of necrotic enteritis by supporting Clostridium perfringens growth. Veterinary Immunology and Immunopathology, 122(1-2), 104-115. doi:10.1016/j.vetimm.2007.10.014

Cooper, K. K., Trinh, H. T., & Songer, J. G. (2009). Immunization with recombinant alpha toxin partially protects broiler chicks against experimental challenge with Clostridium perfringens. Veterinary microbiology, 133(1-2), 92-97.

Costa, M. C., Bessegatto, J. A., Alfieri, A. A., Weese, J. S., Filho, J. A. B., & Oba, A. (2017). Different antibiotic growth promoters induce specific changes in the cecal microbiota membership of broiler chicken. Plos One, 12(2). doi: 10.1371/journal.pone.0171642

Cowieson, A. J., Livingston, M. L., Nogal, B., Hoang, V., Crespo, R., & Livingston, K. A. (2020). Effect of coccidial challenge and vaccination on the performance, veterinary postmortem scores, and blood biochemistry of broiler chickens. Poultry Science, 99(8), 3831-3840.

Craven, S. E. (2000). Colonization of the intestinal tract by Clostridium perfringens and fecal shedding in diet-stressed and unstressed broiler chickens. Poultry Science, 79(6), 843-849.

Cuccato, M., Rubiola, S., Giannuzzi, D., Grego, E., Pregel, P., Divari, S., & Cannizzo, F. T. (2021). 16S rRNA sequencing analysis of the gut microbiota in broiler chickens prophylactically administered with antimicrobial agents. Antibiotics, 10(2), 146.

Dahiya, J. P., Wilkie, D. C., Van Kessel, A. G., & Drew, M. D. (2006). Potential strategies for controlling necrotic enteritis in broiler chickens in post-antibiotic era. Animal Feed Science and Technology, 129(1-2), 60-88.

Darwish, N., Shao, J., Schreier, L. L., & Proszkowiec-Weglarz, M. (2021). Choice of 16S ribosomal RNA primers affects the microbiome analysis in chicken ceca. Scientific Reports, 11(1), 1-15.

De Gussem, M. (2007, August). Coccidiosis in poultry: review on diagnosis, control, prevention and interaction with overall gut health. In Proceedings of the 16th European Symposium on Poultry Nutrition (pp. 253-261). Proceedings of the 16th European Symposium on Poultry Nutrition.

De Maesschalck, C., Eeckhaut, V., Maertens, L., De Lange, L., Marchal, L., Nezer, C., ... & Van Immerseel, F. (2015). Effects of xylo-oligosaccharides on broiler chicken performance and microbiota. Applied and Environmental Microbiology, 81(17), 5880-5888.

Denbow, D. M. (2015). Gastrointestinal anatomy and physiology. In *Sturkie's avian physiology* (pp. 337-366). Academic Press.

Di Bella, J. M., Bao, Y., Gloor, G. B., Burton, J. P., & Reid, G. (2013). High throughput sequencing methods and analysis for microbiome research. Journal of microbiological methods, 95(3), 401-414.

Drew, M. D., Syed, N. A., Goldade, B. G., Laarveld, B., & Van Kessel, A. G. (2004). Effects of dietary protein source and level on intestinal populations of Clostridium perfringens in broiler chickens. Poultry science, 83(3), 414-420.

Ducatelle, R., Eeckhaut, V., Haesebrouck, F., & Van Immerseel, F. (2015). A review on prebiotics and probiotics for the control of dysbiosis: Present status and future perspectives. Animal, 9(1), 43-48. doi:10.1017/S1751731114002584

Fasina, Y. O., Newman, M. M., Stough, J. M., & Liles, M. R. (2016). Effect of Clostridium perfringens infection and antibiotic administration on microbiota in the small intestine of broiler chickens. Poultry Science, 95(2), 247–260. doi: 10.3382/ps/pev329

Fernandes da Costa, S. P., Mot, D., Geeraerts, S., Bokori-Brown, M., Van Immerseel, F., & Titball, R. W. (2016). Variable protection against experimental broiler necrotic enteritis after immunization with the C-terminal fragment of Clostridium perfringens alpha-toxin and a non-toxic NetB variant. Avian Pathology, 45(3), 381-388.

Froebel, L. K., Jalukar, S., Lavergne, T. A., Lee, J. T., & Duong, T. (2019). Administration of dietary prebiotics improves growth performance and reduces pathogen colonization in broiler chickens. Poultry science, 98(12), 6668-6676.

Fuller, R. (1989). Probiotics in man and animals. The Journal of applied bacteriology, 66(5), 365-378.

Gerstein, M. B., Bruce, C., Rozowsky, J. S., Zheng, D., Du, J., Korbel, J. O., ... & Snyder, M. (2007). What is a gene, post-ENCODE? History and updated definition. Genome research, 17(6), 669-681.

Gharib-Naseri, K., Kheravii, S. K., Keerqin, C., Morgan, N., Swick, R. A., Choct, M., & Wu, S. B. (2019). Two different Clostridium perfringens strains produce different levels of necrotic enteritis in broiler chickens. Poultry science, 98(12), 6422-6432.

Gholami-Ahangaran, M., Ahmadi-Dastgerdi, A., & Karimi-Dehkordi, M. (2020). Thymol and carvacrol; as antibiotic alternative in green healthy poultry production. Plant Biotechnology Persa, 2(1), 22-25.

Glendinning, L., Watson, K. A., & Watson, M. (2019). Development of the duodenal, ileal, jejunal and caecal microbiota in chickens. Animal microbiome, 1(1), 1-11.

Godley, A. C., & Hamilton, S. (2020). Different expectations: A comparative history of structure, experience, and strategic alliances in the US and UK poultry sectors, 1920–1990. *Strategic Entrepreneurship Journal*, *14*(1), 89-104.

Gong, J., Si, W., Forster, R. J., Huang, R., Yu, H., Yin, Y., ... & Han, Y. (2007). 16S rRNA gene-based analysis of mucosa-associated bacterial community and phylogeny in the chicken gastrointestinal tracts: from crops to ceca. FEMS microbiology ecology, 59(1), 147-157.

Guban, J., Korver, D. R., Allison, G. E., & Tannock, G. W. (2006). Relationship of dietary antimicrobial drug administration with broiler performance, decreased population levels of Lactobacillus salivarius, and reduced bile salt deconjugation in the ileum of broiler chickens. Poultry science, 85(12), 2186-2194.

Guilloteau, P., Martin, L., Eeckhaut, V., Ducatelle, R., Zabielski, R., & Immerseel, F. V. (2010). From the gut to the peripheral tissues: The multiple effects of butyrate. Nutrition Research Reviews, 23(2), 366-384. doi:10.1017/s0954422410000247

Hall, E., Wodi, A. P., Hamborsky, J., Morelli, V., & Schillie, S. (2021). Epidemiology and Prevention of Vaccine-Preventable Diseases. Washington DC: Public health foundation.

Helmboldt, C. F., & Bryant, E. S. (1971). The pathology of necrotic enteritis in domestic fowl. Avian Diseases, 775-780.

Hofacre, C. L., Mathis, G. F., Lumpkins, B. S., Sygall, R., Vaessen, S., Hofacre, C. S., ... & Clanton, E. (2020). Efficacy of Butyric and Valeric Acid Esters in a Necrotic Enteritis Challenge Model. Avian Diseases, 64(3), 407-414.

Ijaz, U. Z., Sivaloganathan, L., McKenna, A., Richmond, A., Kelly, C., Linton, M., ... & Gundogdu, O. (2018). Comprehensive longitudinal microbiome analysis of the chicken cecum reveals a shift from competitive to environmental drivers and a window of opportunity for Campylobacter. Frontiers in microbiology, 2452.

Johnson, J. S., Spakowicz, D. J., Hong, B. Y., Petersen, L. M., Demkowicz, P., Chen, L., ... & Weinstock, G. M. (2019). Evaluation of 16S rRNA gene sequencing for species and strain-level microbiome analysis. Nature communications, 10(1), 1-11.

Keyburn, A. L., Boyce, J. D., Vaz, P., Bannam, T. L., Ford, M. E., Parker, D., ... & Moore, R. J. (2008). NetB, a new toxin that is associated with avian necrotic enteritis caused by Clostridium perfringens. PLoS pathogens, 4(2), e26.

Kim, M., Morrison, M., & Yu, Z. (2011). Evaluation of different partial 16S rRNA gene sequence regions for phylogenetic analysis of microbiomes. Journal of microbiological methods, 84(1), 81-87

Kiu, R., Brown, J., Bedwell, H., Leclaire, C., Caim, S., Pickard, D., ... & Hall, L. J. (2019). Genomic analysis on broiler-associated Clostridium perfringens strains and exploratory caecal microbiome investigation reveals key factors linked to poultry necrotic enteritis. Animal microbiome, 1(1), 1-14.

Kogut, M. H. (2019). The effect of microbiome modulation on the intestinal health of poultry. Animal feed science and technology, 250, 32-40.

Koutsos, E. A., & Arias, V. J. (2006). Intestinal ecology: Interactions among the gastrointestinal tract, nutrition, and the microflora. Journal of Applied Poultry Research, 15(1), 161-173.

Krogdahl, Å. (1985). Digestion and absorption of lipids in poultry. The Journal of nutrition, 115(5), 675-685.

Lan, P. T. N., Hayashi, H., Sakamoto, M., & Benno, Y. (2002). Phylogenetic analysis of cecal microbiota in chicken by the use of 16S rDNA clone libraries. Microbiology and immunology, 46(6), 371-382.

Latorre, J. D., Adhikari, B., Park, S. H., Teague, K. D., Graham, L. E., Mahaffey, B. D., … Tellez, G. (2018). Evaluation of the Epithelial Barrier Function and Ileal Microbiome in an Established Necrotic Enteritis Challenge Model in Broiler Chickens. Frontiers in Veterinary Science, 5. doi: 10.3389/fvets.2018.00199

Layton, S. L., Hernandez-Velasco, X., Chaitanya, S., Xavier, J., Menconi, A., Latorre, J. D., ... & Tellez, G. (2013). The effect of a Lactobacillus-based probiotic for the control of necrotic enteritis in broilers. Food and nutrition sciences, 1-7.

Lee, J. T., Eckert, N. H., Ameiss, K. A., Stevens, S. M., Anderson, P. N., Anderson, S. M., ... & Caldwell, D. J. (2011). The effect of dietary protein level on performance characteristics of coccidiosis vaccinated and nonvaccinated broilers following mixed-species Eimeria challenge. Poultry Science, 90(9), 1916-1925.

Li, J., Hou, Y., Yi, D., Zhang, J., Wang, L., Qiu, H., ... & Gong, J. (2015). Effects of tributyrin on intestinal energy status, antioxidative capacity and immune response to lipopolysaccharide challenge in broilers. Asian-Australasian Journal of Animal Sciences, 28(12), 1784.

López-Osorio, S., Chaparro-Gutiérrez, J. J., & Gómez-Osorio, L. M. (2020). Overview of poultry Eimeria life cycle and host-parasite interactions. Frontiers in Veterinary Science, 7, 384.

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

Lu, J., Hofacre, C., Smith, F., & Lee, M. D. (2008). Effects of feed additives on the development on the ileal bacterial community of the broiler chicken. Animal, 2(5), 669-676.

Lu, J., Idris, U., Harmon, B., Hofacre, C., Maurer, J. J., & Lee, M. D. (2003). Diversity and succession of the intestinal bacterial community of the maturing broiler chicken. Applied and environmental microbiology, 69(11), 6816-6824.

Lum, J., Sygall, R., & Ros Felip, J. M. (2018). Comparison of tributyrin and coated sodium butyrate as sources of butyric acid for improvement of growth performance in Ross 308 broilers. International Journal of Poultry Science, 17(6), 290-294.

Macdonald, S. E., Nolan, M. J., Harman, K., Boulton, K., Hume, D. A., Tomley, F. M., ... & Blake, D. P. (2017). Effects of Eimeria tenella infection on chicken caecal microbiome diversity, exploring variation associated with severity of pathology. PLoS one, 12(9), e0184890.

Mancabelli, L., Ferrario, C., Milani, C., Mangifesta, M., Turroni, F., Duranti, S., ... & Ventura, M. (2016). Insights into the biodiversity of the gut microbiota of broiler chickens. Environmental microbiology, 18(12), 4727-4738.

Mohd Shaufi, M. A., Sieo, C. C., Chong, C. W., Gan, H. M., & Ho, Y. W. (2015). Deciphering chicken gut microbial dynamics based on high-throughput 16S rRNA metagenomics analyses. Gut pathogens, 7(1), 1-12.

Moore, R. J. (2016). Necrotic enteritis predisposing factors in broiler chickens. Avian Pathology, 45(3), 275-281.

Mora, Z. V. D. L., Macías-Rodríguez, M. E., Arratia-Quijada, J., Gonzalez-Torres, Y. S., Nuño, K., & Villarruel-López, A. (2020). Clostridium perfringens as foodborne pathogen in broiler production: pathophysiology and potential strategies for controlling necrotic enteritis. Animals, 10(9), 1718.

Morey, M., Fernández-Marmiesse, A., Castiñeiras, D., Fraga, J. M., Couce, M. L., & Cocho, J. A. (2013). A glimpse into past, present, and future DNA sequencing. Molecular genetics and metabolism, 110(1-2), 3-24.

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

Nakphaichit, M., Thanomwongwattana, S., Phraephaisarn, C., Sakamoto, N., Keawsompong, S., Nakayama, J., & Nitisinprasert, S. (2011). The effect of including Lactobacillus reuteri KUB-AC5 during post-hatch feeding on the growth and ileum microbiota of broiler chickens. Poultry science, 90(12), 2753-2765.

Namkung, H., Yu, H., Gong, J., & Leeson, S. (2011). Antimicrobial activity of butyrate glycerides toward Salmonella Typhimurium and Clostridium perfringens. Poultry Science, 90(10), 2217-2222.

National Chicken Council. (2020, September). National Chicken Council Animal Welfare Guidelines and Audit Checklist For Broilers. nationalchickencouncil.org. Retrieved March 18, 2022, from https://www.nationalchickencouncil.org/wp-content/uploads/2021/02/NCC-Animal-Welfare-Guidelines_Broilers_Sept2020.pdf

National Chicken Council. (2022, February 15). U.S. broiler performance. Retrieved March 18, 2022, from https://www.nationalchickencouncil.org/about-the-industry/statistics/u-s-broiler-performance/

National Research Council. (1994). Nutrient Requirements of Poultry: Ninth Revised Edition, 1994. Washington, DC: The National Academies Press.

Nilakanta, H., Drews, K. L., Firrell, S., Foulkes, M. A., & Jablonski, K. A. (2014). A review of software for analyzing molecular sequences. BMC Research Notes, 7(1), 1-9.

Oakley, B. B., Buhr, R. J., Ritz, C. W., Kiepper, B. H., Berrang, M. E., Seal, B. S., & Cox, N. A. (2014). Successional changes in the chicken cecal microbiome during 42 days of growth are independent of organic acid feed additives. BMC veterinary research, 10(1), 1-8.

Ocejo, M., Oporto, B., & Hurtado, A. (2019). 16S rRNA amplicon sequencing characterization of caecal microbiome composition of broilers and free-range slow-growing chickens throughout their productive lifespan. Scientific reports, 9(1), 1-14.

Olkowski, A. A., Wojnarowicz, C., Chirino-Trejo, M., & Drew, M. D. (2006). Responses of broiler chickens orally challenged with Clostridium perfringens isolated from field cases of necrotic enteritis. Research in veterinary science, 81(1), 99-108.

Onrust, L., Baeyen, S., Haesebrouck, F., Ducatelle, R., & Van Immerseel, F. (2020). Effect of in feed administration of different butyrate formulations on Salmonella Enteritidis colonization and cecal microbiota in broilers. Veterinary Research, 51(1), 1-15.

Pan, D., & Yu, Z. (2014). Intestinal microbiome of poultry and its interaction with host and diet. Gut microbes, 5(1), 108-119.

Phong, S. F., Shanmugavelu, S., Thayalini, K., Noraini, S., & Wong, H. K. (2010). Detection of Lactobacillus, Bacteroides and Clostridium perfringens in the gastrointestinal contents of chicken fed different diets by real-time PCR. J. Trop. Agric. Food Sci, 38, 81-87.

Plöger, S., Stumpff, F., Penner, G. B., Schulzke, J. D., Gäbel, G., Martens, H., ... & Aschenbach, J. R. (2012). Microbial butyrate and its role for barrier function in the gastrointestinal tract. Annals of the New York academy of sciences, 1258(1), 52-59.

Reuben, R. C., Roy, P. C., Sarkar, S. L., Alam, R. U., & Jahid, I. K. (2019). Isolation, characterization, and assessment of lactic acid bacteria toward their selection as poultry probiotics. BMC microbiology, 19(1), 1-20.

Riaz, A., Umar, S., Munir, M. T., & Tariq, M. (2017). Replacements of antibiotics in the control of necrotic enteritis: a review. Sci Lett, 5(3), 208-216.

Rinttilä, T., & Apajalahti, J. (2013). Intestinal microbiota and metabolites—Implications for broiler chicken health and performance. Journal of Applied Poultry Research, 22(3), 647-658.

Rychlik, I. (2020). Composition and function of chicken gut microbiota. Animals, 10(1), 103.

Schloss, P. D. (2010). The effects of alignment quality, distance calculation method, sequence filtering, and region on the analysis of 16S rRNA gene-based studies. PLoS computational biology, 6(7), e1000844.

Seidavi, A., Mirhosseini, S. Z., Shivazad, M., Chamani, M., Sadeghi, A. A., & Pourseify, R. (2010). Detection and investigation of Escherichia coli in contents of duodenum, jejunum, ileum and cecum of broilers at different ages by PCR. Asia Pacific Journal of Molecular Biology and Biotechnology, 18, 321-326.

Sergeant, M. J., Constantinidou, C., Cogan, T. A., Bedford, M. R., Penn, C. W., & Pallen, M. J. (2014). Extensive microbial and functional diversity within the chicken cecal microbiome. PloS one, 9(3), e91941.

Shendure, J. A., Porreca, G. J., & Church, G. M. (2008). Overview of DNA sequencing strategies. Current Protocols in Molecular Biology, 81(1), 7-1.

Shendure, J., Balasubramanian, S., Church, G. M., Gilbert, W., Rogers, J., Schloss, J. A., & Waterston, R. H. (2017). DNA sequencing at 40: past, present and future. Nature, 550(7676), 345-353.

Shojadoost, B., Vince, A. R., & Prescott, J. F. (2012). The successful experimental induction of necrotic enteritis in chickens by Clostridium perfringens: A critical review. Veterinary Research, 43(1), 74. doi:10.1186/1297-9716-43-74

Shokryazdan, P., Faseleh Jahromi, M., Liang, J. B., Ramasamy, K., Sieo, C. C., & Ho, Y. W. (2017). Effects of a Lactobacillus salivarius mixture on performance, intestinal health and serum lipids of broiler chickens. PLoS one, 12(5), e0175959.

Sotira, S., Dell'Anno, M., Caprarulo, V., Hejna, M., Pirrone, F., Callegari, M. L., ... & Rossi, L. (2020). Effects of tributyrin supplementation on growth performance, insulin, blood metabolites and gut microbiota in weaned piglets. Animals, 10(4), 726.

Stanley, D., Hughes, R. J., & Moore, R. J. (2014). Microbiota of the chicken gastrointestinal tract: influence on health, productivity and disease. Applied microbiology and biotechnology, 98(10), 4301-4310.

Stanley, D., Keyburn, A. L., Denman, S. E., & Moore, R. J. (2012). Changes in the caecal microflora of chickens following Clostridium perfringens challenge to induce necrotic enteritis. Veterinary microbiology, 159(1-2), 155-162.

Stewart, A. S., Pratt-Phillips, S., & Gonzalez, L. M. (2017). Alterations in intestinal permeability: the role of the "leaky gut" in health and disease. Journal of equine veterinary science, 52, 10-22.

Sureshkumar, S., Lee, H. C., Jung, S. K., Kim, D., Oh, K. B., Yang, H., ... & Byun, S. J. (2021). Inclusion of Lactobacillus salivarius strain revealed a positive effect on improving growth performance, fecal microbiota and immunological responses in chicken. Archives of Microbiology, 203(2), 847-853.

Svihus, B., Choct, M., & Classen, H. L. (2013). Function and nutritional roles of the avian caeca: a review. *World's Poultry Science Journal*, *69*(2), 249-264.

Takehara, M., Takagishi, T., Seike, S., Ohtani, K., Kobayashi, K., Miyamoto, K., ... & Nagahama, M. (2016). Clostridium perfringens α-toxin impairs innate immunity via inhibition of neutrophil differentiation. Scientific reports, 6(1), 1-12.

Tamirat, B., Tamirat, H., Bassazin, G., Alemayhu, T., & Tadesse, M. (2017). Review on necrotic enteritis. British Journal of Poultry Sciences, 6(3), 63-72.

Tsiouris, V., Georgopoulou, I., Batzios, C., Pappaioannou, N., Ducatelle, R., & Fortomaris, P. (2015a). The effect of cold stress on the pathogenesis of necrotic enteritis in broiler chicks. Avian Pathology, 44(6), 430-435.

Tsiouris, V., Georgopoulou, I., Batzios, C., Pappaioannou, N., Ducatelle, R., & Fortomaris, P. (2015b). High stocking density as a predisposing factor for necrotic enteritis in broiler chicks. Avian Pathology, 44(2), 59-66.

Tsiouris, V. (2016). Poultry management: a useful tool for the control of necrotic enteritis in poultry. Avian Pathology, 45(3), 323-325.

Tsiouris, V., Georgopoulou, I., Batzios, C., Pappaioannou, N., Ducatelle, R., & Fortomaris, P. (2018). Heat stress as a predisposing factor for necrotic enteritis in broiler chicks. Avian Pathology, 47(6), 616-624.

Van Immerseel, F., De Buck, J., Pasmans, F., Velge, P., Bottreau, E., Fievez, V., ... & Ducatelle, R. (2003). Invasion of Salmonella enteritidis in avian intestinal epithelial cells in vitro is influenced by short-chain fatty acids. International journal of food microbiology, 85(3), 237-248.

Wade, B., Keyburn, A. L., Haring, V., Ford, M., Rood, J. I., & Moore, R. J. (2016). The adherent abilities of Clostridium perfringens strains are critical for the pathogenesis of avian necrotic enteritis. Veterinary microbiology, 197, 53-61.

Wang, C., Shen, Z., Cao, S., Zhang, Q., Peng, Y., Hong, Q., ... & Hu, C. (2019). Effects of tributyrin on growth performance, intestinal microflora and barrier function of weaned pigs. Animal Feed Science and Technology, 258, 114311.

Wang, H. B., Wang, P. Y., Wang, X., Wan, Y. L., & Liu, Y. C. (2012). Butyrate enhances intestinal epithelial barrier function via up-regulation of tight junction protein Claudin-1 transcription. Digestive diseases and sciences, 57(12), 3126-3135.

Wang, J., Fan, H., Han, Y., Wei, J., Zhao, J., & Zhou, Z. (2016). Pyrosequencing of the broiler chicken gastrointestinal tract reveals the regional similarity and dissimilarity of microbial community. Canadian Journal of Animal Science, 97(2), 302-313.

Wang, J., Ishfaq, M., Guo, Y., Chen, C., & Li, J. (2020). Assessment of probiotic properties of Lactobacillus salivarius isolated from chickens as feed additives. Frontiers in veterinary science, 415.

Wang, Z., Zeng, X., Mo, Y., Smith, K., Guo, Y., & Lin, J. (2012). Identification and characterization of a bile salt hydrolase from Lactobacillus salivarius for development of novel alternatives to antibiotic growth promoters. Applied and environmental microbiology, 78(24), 8795-8802.

Wei, S., Morrison, M., & Yu, Z. (2013). Bacterial census of poultry intestinal microbiome. Poultry science, 92(3), 671-683

Wilkie, D. C., Van Kessel, A. G., White, L. J., Laarveld, B., & Drew, M. D. (2005). Dietary amino acids affect intestinal Clostridium perfringens populations in broiler chickens. Canadian Journal of Animal Science, 85(2), 185-193.

Williams, R. B. (2005). Intercurrent coccidiosis and necrotic enteritis of chickens: rational, integrated disease management by maintenance of gut integrity. Avian pathology, 34(3), 159-180.

Wu, S. B., Stanley, D., Rodgers, N., Swick, R. A., & Moore, R. J. (2014). Two necrotic enteritis predisposing factors, dietary fishmeal and Eimeria infection, induce large changes in the caecal microbiota of broiler chickens. Veterinary microbiology, 169(3-4), 188-197.

Wu, Y., Zhen, W., Geng, Y., Wang, Z., & Guo, Y. (2019). Pretreatment with probiotic Enterococcus faecium NCIMB 11181 ameliorates necrotic enteritis-induced intestinal barrier injury in broiler chickens. Scientific reports, 9(1), 1-17.

Xiao, Y., Xiang, Y., Zhou, W., Chen, J., Li, K., & Yang, H. (2017). Microbial community mapping in intestinal tract of broiler chicken. Poultry science, 96(5), 1387-1393.

Xu, S., Lin, Y., Zeng, D., Zhou, M., Zeng, Y., Wang, H., ... & Ni, X. (2018). Bacillus licheniformis normalize the ileum microbiota of chickens infected with necrotic enteritis. Scientific reports, 8(1), 1-10.

Yadav, D. K., Yadav, N., & Khurana, S. M. P. (2020). Vaccines: present status and applications. In Animal biotechnology (pp. 523-542). Academic Press.

Yeoman, C. J., Chia, N., Jeraldo, P., Sipos, M., Goldenfeld, N. D., & White, B. A. (2012). The microbiome of the chicken gastrointestinal tract. Animal health research reviews, 13(1), 89-99.

Zahoor, I., Ghayas, A., & Basheer, A. (2018). Genetics and genomics of susceptibility and immune response to necrotic enteritis in chicken: a review. Molecular biology reports, 45(1), 31-37.

Zaragoza, N. E., Orellana, C. A., Moonen, G. A., Moutafis, G., & Marcellin, E. (2019). Vaccine production to protect animals against pathogenic clostridia. Toxins, 11(9), 525.

Zhou, H., Gong, J., Brisbin, J., Yu, H., Sanei, B., Sabour, P., & Sharif, S. (2007). Appropriate Chicken Sample Size for Identifying the Composition of Broiler Intestinal Microbiota Affected by Dietary Antibiotics, Using the Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis Technique. Poultry Science, 86(12), 2541–2549. doi: 10.3382/ps.2007-00267

Zhu, X. Y., Zhong, T., Pandya, Y., & Joerger, R. D. (2002). 16S rRNA-based analysis of microbiota from the cecum of broiler chickens. Applied and environmental microbiology, 68(1), 124-137.

CHAPTER II.

Intestinal Microbiota Analysis of Broiler Chickens under Necrotic Enteritis Challenge and Tributyrin Supplementation

1. Introduction

The overwhelming human desire to eat chicken led to advancements in animal breeding, operations management, and nutrition that revolutionized the poultry industry over a half-century period. These practices combined to produce what is now an exceptional resource- and cost-efficient animal-derived protein production model (Putman et al., 2017; Gerber et al.,2007). This sophisticated system heralded by the poultry industry relies on excellent feed efficiency, low mortality, and a plethora of live, healthy birds to meet the dietary needs of billions of hungry humans while remaining profitable in the market sector. However, a threat to maintaining this efficient status has emerged, potentially changing the management of broiler chickens in the future.

For decades (Gerber et al., 2007), antibiotic growth promoters (AGPs) like the *Streptomyces virginieae* - derived virginiamycin (George et al., 1982), have been used to enhance bird performance and control disease outbreaks. After the identification of *Enterococcus faecium* strains showing resistance to the human antibiotic, quinupristin-dalfopristin (Dumonceaux et al., 2006) and the increasing possibility of creating other dangerous, antibiotic-resistant strains of bacteria, the European Union banned the use of non-therapeutic antibiotics in animal production in 2006 (Huyghebaert et al., 2011; Caly et al., 2015). Recently, in the United States, the use of antibiotics in virtually any livestock industry in any scenario has become a consumer taboo, pushing producers to abandon antibiotics altogether (Huyghebaert et al., 2011). The removal of antibiotics from poultry production has led to the re-emergence of a serious gastrointestinal disease, NE that is catalyzed by the pathogen, *Clostridium perfringens* (Shojadoost et al., 2012; M'sadeq et al., 2015).

NE in chickens occurs in two forms, clinical and subclinical, both of which negatively affect animal welfare and profitability. The clinical version causes anorexia, erosion of intestinal mucosa, diarrhea, and death (M'Sadeq, et al., 2015). Sub-clinical infection poses mild disease symptoms and diminishes animal performance, thereby creating an opportunity for economic losses (Timbermont et al., 2010). A subclinical infection also poses an increased threat of foodborne pathogen transmission to humans due to the increased risk of *C. perfringens* contamination during processing (Immerseel, et al., 2004). Neither form of NE is desirable whether you are a chicken producer, a chicken consumer, or a chicken, so eliminating the disease burden comes with benefits from all perspectives. Although NE was once easily prevented and controlled with antibiotic growth promoters (AGPs) like virginiamycin (George et al., 1982; Williams, 2005), lincomycin, or bacitracin (Williams, 2005), these treatments can no longer be used as a preventative measure. We must turn our attention to other areas of animal health and disease management if we hope to solve the NE puzzle without antibiotics.

The search for an AGP replacement to combat NE within broiler production is not uncharted territory, but so far has yielded no single, definitive solution. Previous reviews (Huyghebaert et al., 2011) outlining the potential for different substances to replace AGPs as feed additives, have described organic acids, including the short-chain fatty acid (SCFA), butyric acid, as a partial solution due to their array of beneficial host effects. Naturally occurring butyrate serves as an energy source for epithelial cells in the GIT, helps regulate cell proliferation, maturation, apoptosis, and has been shown to reduce inflammatory cytokine production in humans. Because of its status as a weak acid (pKa <4.8), butyrate is used as a viable pathogen control tactic in food and livestock production, especially against *Salmonella* (Guilloteau et al., 2010). It has been demonstrated that butyrate does not reduce *C. perfringens* colonization directly but has been

shown to reduce the severity and occurrence of necrotic lesions caused by *C. perfringens* in broilers. This is possibly due to butyrate's improvement of intestinal morphology including increased villus height (Timbermont et al., 2010).

Namkung et. al. found that the most effective configuration of butyrate for inhibiting enteric pathogen overload was pure butyric acid. However, butyric acid in its unprotected form is too corrosive and unpalatable to use directly in feed (Li et al., 2015). Furthermore, pure butyric acid is metabolized before it can reach the microbial communities of the small intestine (Moquet et al., 2016). By encapsulating a butyrate salt, sodium butyrate, within a lipid matrix or by anchoring butyrate to glycerides (mono-, di-, and tributyrin), researchers can delay the absorption of butyrate until it has reached the small intestine and reduce the toxic and unpalatable effects of free butyric acid (Li et al., 2015). Any of these chemical compositions of butyric acid are commonly considered "butyrate" or "butyrate supplements" within the literature (Guilloteau et al., 2010), but for this study, we will refer to the use of tributyrin as the butyrate supplement of interest.

Innovative 16S rRNA gene sequencing has given insight into the microbial populations within the body to understand the role of the microbiome in human and animal hosts. The form of amplicon gene sequencing used in our lab utilizes PCR primers to target and amplify the V4 gene region. The resulting amplicons are aligned to a developed gene library database to identify the organisms within the gut community (Chakravorty et al., 2007). After identification, operational taxonomic units (OTUs) are assigned, offering a certainty of at least 97%. This complex procedure is performed in a matter of hours and yields DNA sequences hundreds of base pairs (bp) long, in contrast to the earliest DNA sequencing, which took months to years of lab work to produce sequences only up to one hundred bp long (Heather & Chain, 2016).

Few publications addressing the relationship specifically between butyrate additives and the intestinal microbiota of the host animal exist, which narrows further as the host is specified as broiler chickens (Guilloteau et al., 2010; Bortoluzzi et al., 2019). To our knowledge, this is the first experiment conducted using high-throughput next-generation sequencing (HT-NGS) with the intent to illuminate the shift within the broiler chicken's intestinal microbial communities as a response to tributyrin as a treatment for NE (Latorre et al., 2018; Fasina et al., 2016). The previously demonstrated beneficial effects of butyrate are indisputable, but to date little is known about the effect of tributyrin on the composition of the broiler intestinal biota, and if these effects may help explain the ill-understood, underlying mechanisms that make butyric acid supplements advantageous for gastrointestinal health. If our hypothesis is correct, we will see a microbial shift within the cecum and ileum which promotes intestinal health after the administration of the butyrate treatment during the NE challenge.

2. Materials and Methods

2.1 Reader's Note

This study was a collaborative effort with the Southern Poultry Research Group (SPRG) in which the number of samples used and discussed in this paper is a subset of the samples from birds raised as part of a separate target study. The birds used in this study were randomly selected from the T1, nonmedicated, unchallenged with *C. perfringens* (negative control group), T2, nonmedicated, challenged with *C. perfringens* (positive control group), and T6, treated with butyrin (Butyrin SR130, Perstorp) in the feed at 0.5kg/metric ton from day 0 to day 14 and at 0.25 kg/metric ton from day 14 to 42 (variable dose) and challenged with *C. perfringens,* groups as described in Hofacre, et al., 2020. The goal of our study was to examine the intestinal microbial communities of commercial-type broilers treated with tributyrin under an induced NE

challenge. This study was approved by the Southern Poultry Research Group Institutional Animal Care and Use Committee (IACUC). All information regarding animal rearing, including animal age, housing, and feeding regimen, as well as experimental design, including treatment administration, dosage, and sample collection was obtained via email correspondence with Dr. Charles Hofacre of the Southern Poultry Research Group (C. F. Hofacre, personal communication, July 7, 2020 – November 22, 2021).

2.2 Animal Information, Experimental Design, and Housing

Three thousand *Ross*-708, day-of-hatch, male broiler chicks were procured from the Aviagen Hatchery in Blairsville, Georgia, and transferred to Sanford House 2 at the Southern Poultry Research Group, Inc. in Nicholson, GA. Birds were administered a coccidiosis vaccine (Merck Coccivac-B52, lot number 94320070) via spray cabinet at the manufacturer's recommended dosage upon arrival. Only healthy-appearing birds, including those free from physical defects of the legs, wings, and beak, active and alert in disposition, and without the presence of respiratory disease, were selected for use in this study. The birds were assigned to treatment groups (T1, T2, or T6) and placed into pens at fifty birds per pen, with 10 replicate pens per treatment group. The housing facility was divided into 10 blocks, with each block containing one replicate of each treatment group. Treatments were assigned to pens within blocks using Random Permutation Tables (Hofacre, et al., 2020). Each pen was 1.5 × 3.0 meters with a stocking density of 11 birds per m^2. The solid-sided barn that housed the pens was primarily heated by thermostatically controlled gas heaters as needed, and additionally, one heat lamp per pen was available to provide supplemental heating as needed. Ventilation and cooling of birds were maintained by commercial-type fans. Approximately 10 cm of fresh pine shavings were used as the bedding material atop dirt floors and were not replaced or amended throughout the trial. Likewise, feed

and water were available *ad libitum* and provided by one tube feeder and one bell drinker per pen (50 bird/pen drinker ratio). Animal care practices conformed to the *Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching* (National Research Council, 2010). This portion of the study was performed by members of the Southern Poultry Research Group in Athens, Georgia.

2.3 Experimental Ration

Birds in all groups were fed a common US feedstuff ration consisting of nonmedicated commercial-type broiler crumbled starter and pelleted grower and finisher diets that met NRC guidelines (National Research Council, 1994). The phasing of the three feed types is as follows: Starter (day 0–14), Grower (day 14 – 35), and Finisher (day 35 – 42). Only birds consuming up to the Grower ration were used in this study. This ration was used as the base experimental ration for birds in each treatment group. Before pelleting, specific treatment additives were mixed with the base ration at the SPRG Feed Mill to optimize uniform distribution. The T6 treatment group our study is concerned with also included variable doses of a tributyrin-based product that varied depending on the feeding phase as follows: Starter (0.5 kg/metric ton Butyrin SR130), and Grower/Finisher (0.25 kg/metric ton Butyrin SR130). Pelleting was completed by a California Pellet Mill (80° C). At all times after chick arrival, rations were fed *ad libitum* until termination of the study. On feeding phase change days (day 14, 35, and 42), the unconsumed feed was removed, weighed, and replaced with the next feeding phase ration, except in the Finisher phase, in which feed replacement was unnecessary, as the trial was terminated. Only birds consuming feed until day 21 were used in this study. This portion of the study was performed by members of the Southern Poultry Research Group in Athens, Georgia.

2.4 *Eimeria maxima* and *C. perfringens* Induced Necrotic Enteritis Challenge

2.4.1 E. maxima challenge

It is common for broiler chicks in a production setting to receive a coccidia vaccine on the day of arrival, so a coccidia vaccine (Merck Coccivac-B52, lot number 94320070) was administered on day 0 to all birds, including those in the T1 negative control group. *E. maxima,* well known for causing coccidiosis, a predisposing factor to NE, was introduced to induce an active NE disease challenge on birds in the T2 and T6 groups. In addition, an *E. maxima* challenge was introduced to each pen in the T2 and T6 groups on day 14 by spreading twenty (20) mL of a solution containing *E. maxima* (approx. 5,000 oocysts per bird) in the litter around feeders and drinkers of each pen. This portion of the study was performed by members of the Southern Poultry Research Group in Athens, Georgia.

2.4.2 C. perfringens challenge

Birds in treatment groups T2 and T6 were challenged with *C. perfringens* strain no. 6 (CP6), on days 18 and 19 at a dosage of roughly 1 × 108 CFU per bird. The CP6 strain is a wild type, NetB+, TpeL+, fifteen (15) hour culture, established by Hofacre, et al., 1998, in which a fresh culture was prepared from a stock culture solution and incubated in a thioglycolate broth overnight at 35°C until approximately 10^7-10^8 CFU/mL target was achieved (Hofacre, et al., 2018). The *C. perfringens* challenge vector was a measured amount of water (~125 ml CP6 to 75 ml water) per pen. In groups T2 and T6, all feed and water were removed for three hours prior to the *C. perfringens* challenge, after which time birds in each pen were allowed access to the measured amount of contaminated water for 30 minutes. Afterward, the uncontaminated feed and

water were replaced. This portion of the study was performed by members of the Southern Poultry Research Group in Athens, Georgia.

2.5 Sample Collection of Ileal and Cecal segments

On day 21, three birds per pen, selected by the first-to-hand method, (including those used in this study) were humanely euthanized by cervical disarticulation, necropsied, lesion scored, and the ileum and cecum were harvested. Samples included in this study were taken from birds housed in pens representative of the ten (10) block random design. Ileal and cecal segments roughly 5 cm in length were harvested from fifteen birds per treatment group by choosing either one or two birds at random from each replicate for that treatment. In total, forty-five cecal and forty-five ileal samples were utilized in this study. The ileal and cecum samples were placed into 50 ml polypropylene conical centrifuge tubes and stored on ice for transfer to the University of Arkansas, Fayetteville campus. This portion of the study was performed by members of the Southern Poultry Research Group in Athens, Georgia. Upon arrival at the University of Arkansas, the tubes were stored at -80°C until DNA extraction was performed.

2.6 NE Challenge Confirmation through Intestinal Lesion Scoring

On day 21, approximately twenty-four (24) hours after target mortality of 15% was reached, three (3) birds per pen were humanely euthanized, weighed, necropsied, and examined for gross lesions indicative of NE following an established method of lesion score determination (Hofacre, 1998). Scoring justification is as follows: Lesion score 0 = Normal; Lesion score 1 = Slight mucous covering small intestine; Lesion score 2 = Necrotic small intestine mucosa; Lesion score 3 = Sloughed and bloody small intestine mucosa and contents (Hofacre et al., 1998). This portion

of the study was performed by members of the Southern Poultry Research Group in Athens, Georgia.

2.7 DNA Isolation and Extraction

Ileal and cecal samples were stored at -80°C until the day of the extraction. Samples remained on ice during the content collection and DNA extraction process. Approximately 200 mg of ileal or cecal contents were collected from which DNA was extracted using the DNeasy PowerLyzer® PowerSoil® DNA Isolation Kit (Qiagen, Germantown, MD, USA) according to the manufacturer's protocol. This process included mechanical, chemical, and thermal cell lysis through bead beating, application of an anionic detergent (Sodium Dodecyl Sulfate (SDS)), and heating and cooling steps. Additionally, DNA was filtered from organic and inorganic contaminants using patented reagents (Inhibitor Removal Technology®), centrifugation, and manual separation of supernatant and debris pellet via micropipette. Next, a high-concentration salt solution was applied to allow the binding of DNA to a silica-lined Spin Filter and an ethanol wash was used to rinse non-DNA particles from the sample. Finally, the isolated and cleansed DNA is treated with a buffer solution and centrifuged briefly to remove it from the Spin Filter. The concentration and purity of the resulting DNA extract were measured by a NanoDrop One spectrophotometer (Thermo Fisher Scientific, Madison, WI, USA) at 260 and 280 nm. For uniformity during the downstream sequencing process, each DNA sample was diluted to 10ng/µL with purified, DNase- and RNase-free water. This portion of the study was performed by Taylor McKinney and Robert Story.

2.8 Library Preparation and Primer Selection

The 16S ribosomal RNA gene libraries were constructed following the strategy outlined by Kozich, et al., 2013, for the MiSeq Illumina sequencing platform. The V4 hypervariable region of the 16S rRNA gene found within the bacterial genome was amplified using universal primers U515F and a single-mismatch 806R (F: 5′-GTGCCAGCMGCCGCGGTAA-3′ and R: 5′-GGACTACHVGGGTWTCTAAT-3′) with attaching Illumina sequencing primer and barcode sequence. Pooled amplicons were then sequenced using the High-Throughput Next Generation Sequencing (HT-NGS) Illumina Kit. This pair-end sequencing was completed following Illumina MiSeq protocols (2 × 250 bp, MiSeq Reagent Kit v2, 500 cycles, 20% PhiX) as described in Wang, et al., 2019. In this process, amplicon size was confirmed through gel electrophoresis and amplicons were normalized using the SequelPrep Normalization Plate Kit (Invitrogen, Carlsbad, CA, USA). After normalization of amplicons, the Agilent Bioanalyzer 2100 (Agilent, Santa Clara, CA, USA) was used to assess the quality of amplicons and quantitative RT-PCR was used to assess amplicon quantity. Quality control measures were taken in each MiSeq run, including the use of negative controls from DNA extraction and PCR amplification and a positive control mock community DNA (ZymoBIOMICS™ Microbial Community Standard (Zymo Research, Irvine, CA, USA)) (Wei et al., 2020). This portion of the study was performed by Xiaofan Wang.

2.9 16S rRNA Based Microbiota Data Analysis

Illumina MiSeq fastq sequence reads were imported into the Mothur platform (v1.39.5) and analyzed following the MiSeq standard operating procedures to quality filter the sequences, align with the references SILVA (version 132) database, and cluster sequences into operation taxonomic units (OTUs) (Wei et al., 2020). The OTUs were classified against the Ribosomal

Database Project (RDP) database with a 97% identity threshold. Alpha diversity, the microbial diversity of the intestinal contents within each treatment group (intrasample variation) (Allaband et al., 2019), was evaluated by Shannon index and Observed OTU measures, and tested with Kruskal-Wallis. Beta diversity, the distance between subjects or treatment groups (inter-sample variation) (Allaband et al., 2019), was analyzed via Bray Curtis and Jaccard distances, visualized through Principal Coordinate Analysis. An analysis of similarity (ANOSIM) was performed to evaluate the diversity between treatment groups using Mothur (v1.39.5). Through the galaxy server, linear discriminant analysis effect size analysis (LEfSe) was used to identify specific taxa at the OTU level that were enriched in each treatment group (Segata et. al., 2011) and a linear determinate analysis (LDA) was used to visualize the treatment group OTU enrichment. The Kruskal-Wallis (alpha) value was set at 0.05 and the LDA threshold score indicating a significant difference was set to 2.0. There were no enriched features found in either cecal or ileal samples from the LEfSe test, and no subsequent LDA visualization was obtained. This portion of the study was performed by Jianmin Chai and Taylor McKinney.

Random Forest, a complex decision-tree algorithm, was used to identify microbial signatures that better differentiate between treatment groups and determine the importance of those signatures. A random subset of samples from our study was used to develop the forest of decision trees. To determine the accuracy of the forest's ability to predict correct results, the remaining samples that were not used to create the forest are tested within the forest. The result of this test of the forest's accuracy is called the Out-of-Bag error, which gives the probability that any sample chosen "out of a bag" will be accurately placed into the predicted treatment group. This machine-learning technique assigns an importance score (mean decrease accuracy, MDA) to the microbial signatures (OTUs) based on the increase in error that would result from removing that trait from

predictors. The importance score of each feature was ranked and those with an MDA greater than 3 were considered highly predictive in this study. Random forest models were used to predict taxonomy that classified the ileal and cecal bacterial communities into classes based on the treatment method. The boxplots of the selected features were drawn in R (v3.6.0). This portion of the study was performed by Jianmin Chai.

After Random Forest identified OTUs with the highest MDA score, the corresponding DNA sequences of those OTUs were fed into the NCBI's (National Center for Biotechnology Information) BLAST (Basic Local Alignment Search Tool) program to best identify the DNA sequence with a known organism within the database. The standard nucleotide BLAST (blastn suite) program was selected for sequence comparison to the selected database "16S ribosomal RNA Sequences (Bacteria and Archaea), and all other parameters were left at default. This portion of the study was performed by Taylor McKinney.

3. Results

3.1 Growth Performance

The growth performance of birds in our three treatment groups was previously reported in Hofacre, C. L., et al., 2020, as part of their original, separate study. The information relevant to this project included weight gain, feed conversion ratio, lesion scoring, and mortality rate up to day 21. Hofacre, C. L., et al., found that, at 14 days of age, the T6 group had the highest weight gain and lowest adjusted feed conversion ratio and the T1 and T2 groups were not different. On day 20, the T2 positive challenge control group had the highest lesion scores and the T1 group had the lowest lesion scores (Hofacre, C.L., et al., 202). Additionally, birds in the T2 group reached a 15% mortality on day 21, prompting the termination of the study. This information

was used to verify the success of the NE challenge for further study of the intestinal microbiota and its relationship to NE and tributyrin supplementation in our project.

3.2 Effect of *C. perfringens* challenge and Tributyrin treatment on Microbiota

Alpha and Beta Diversity

The microbiota species evenness (Shannon index, Figure 1. a,b) and richness (Observed OTUs, Figure 1. c,d) values depicting alpha diversity of the T2 or T6 groups taken from ileal or cecal samples were not significantly influenced by the NE challenge or variable-dose butyrin supplementation. The richness or number of OTUs observed within the ileum and cecum, remained stable regardless of treatment type. Numerically, the T2 group of ileal samples exhibited fewer OTU counts than the negative control (T1) and T6 groups. Likewise, the ileal T1 group numerically exhibited fewer OTU counts than the ileal T6 treatment group. The cecal samples numerically showed little difference between the T1 and T2 groups, but the T6 treatment group exhibited greater OTU counts than both other groups. Beta diversity measures Bray Curtis (Figure 2. a,b) and Jaccard (Figure 2. c,d) reveal no significant changes in gut community structure and composition among the three treatment groups of the cecal or ileal samples after *C. perfringens* challenge or tributyrin supplementation.

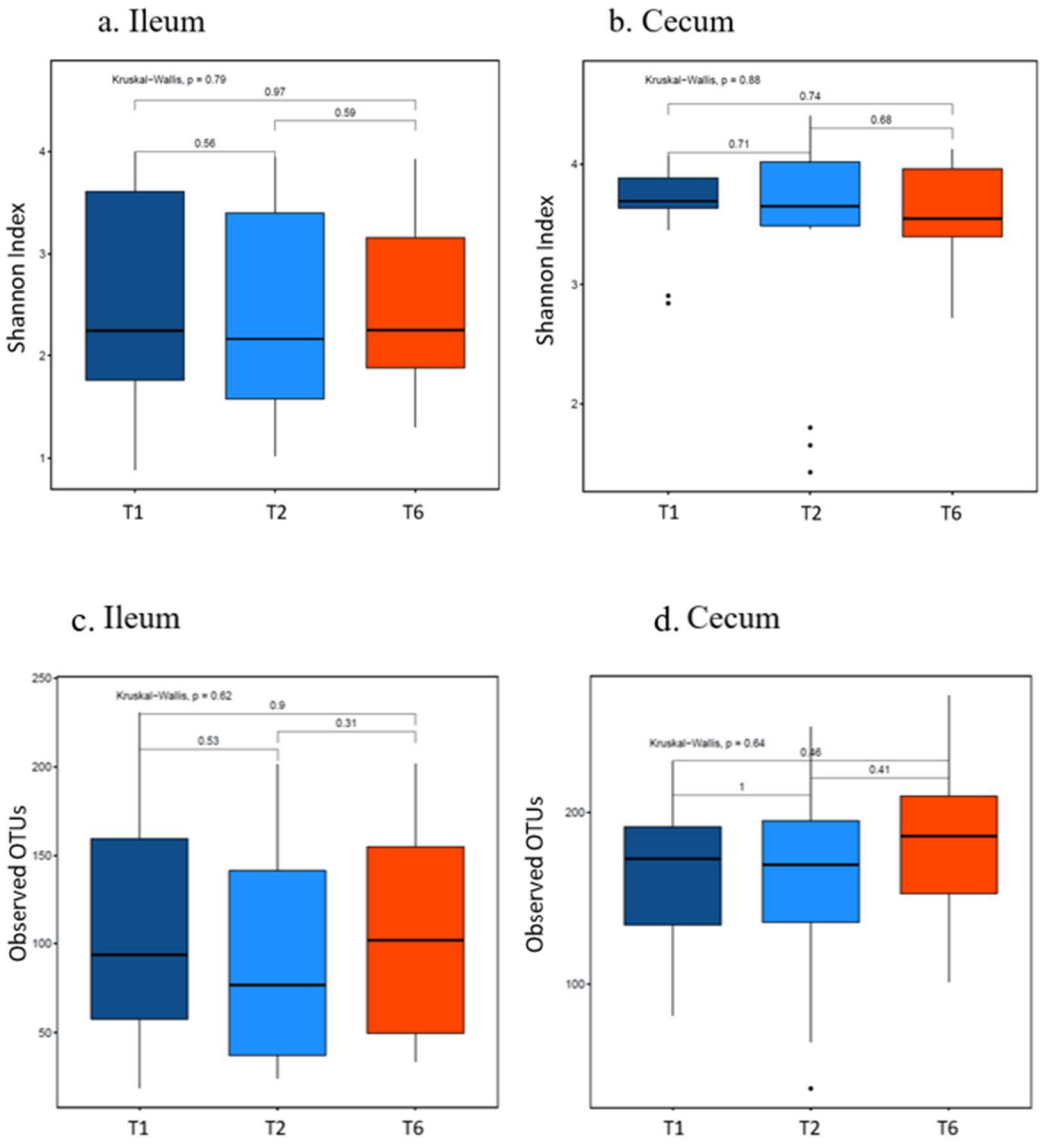

Figure 1. Boxplot of alpha diversity indices representing evenness (a,b) and richness (c,d) of the microbial communities of the negative control (T1), necrotic enteritis challenge (T2), and necrotic enteritis challenge with tributyrin treatment (T6) groups in the ileum (a,c) and cecum (b,d) of broiler chickens, tested with Kruskal-Wallis.

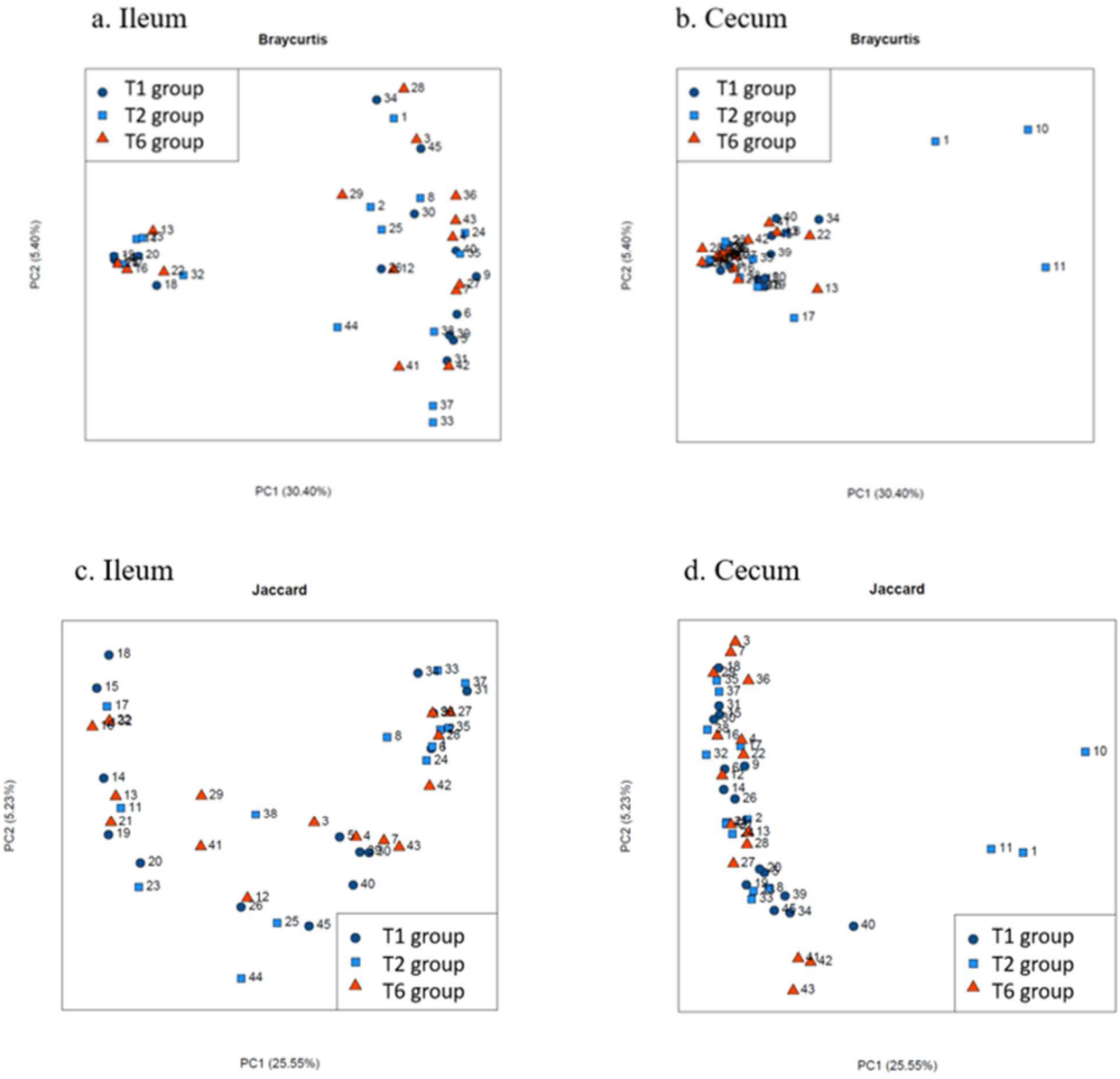

Figure 2. Principal Coordinate Analysis (PCoA) plot of Braycurtis (a,b) and Jaccard (c,d) Beta Diversity measures representing microbial community similarity of the negative control (T1), necrotic enteritis challenge (T2), and necrotic enteritis challenge with tributyrin treatment (T6) groups in the ileum (a,c) and cecum (b,d) of broiler chickens. Numbers beside plot points represent animal identification number.

3.3 Relative Abundance of Ileal and Cecal Phyla

In the cecum, Firmicutes account for more than 70 percent of the total abundance of microbial species, and in the ileum, this rises to more than 80 percent. Our results, however, showed no significant difference ($p<0.05$, LDA score >2.0) in relative abundance among treatment groups for ileal (Figure 3. a) or cecal (Figure 3. b) samples at the phyla level. The relative abundance of *Firmicutes* in the T2 and T6 groups of ileal samples were statistically indistinguishable but numerically greater than in the T1 group. An increase in the relative abundance of taxa from the Bacteroidetes phyla was observed numerically for the T1 group of ileal samples, however, this was not statistically significant. These OTUs include Bacteria, Actinobacteria, and Proteobacteria, all commonly found along the ileum segment of the intestinal tract of broiler chickens (Wei et al., 2013; Mohd Shaufi et al., 2015; Xiao et al., 2017). The cecal samples from our study provided similar results. A numerical decrease in Bacteroidetes was observed in the T2 group of cecal samples when compared to the T1 and T6 groups, and the remaining OTUs of the Bacteria, Actinobacteria, and Proteobacteria phyla were relatively stable and statistically similar among treatment groups ($p<0.05$).

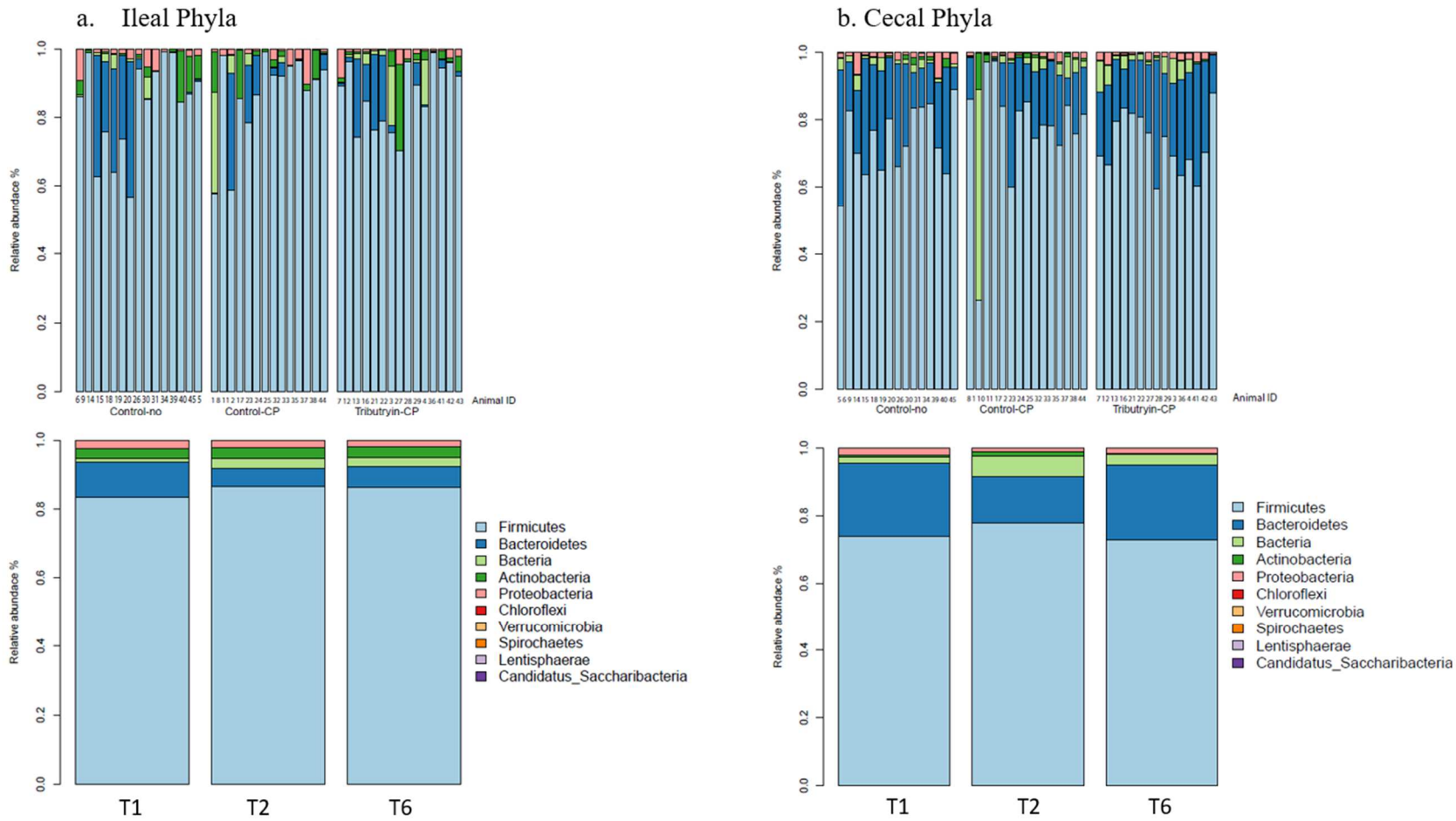

Figure 3. Stacked bar charts of relative abundance of ileal (a) and cecal (b) phyla found in the T1 (negative control), T2 (non-medicated, *C. perfringens* and *E. maxima* challenged), and T6 (*C. perfringens* and *E. maxima* challenged, tributyrin supplemented) groups.

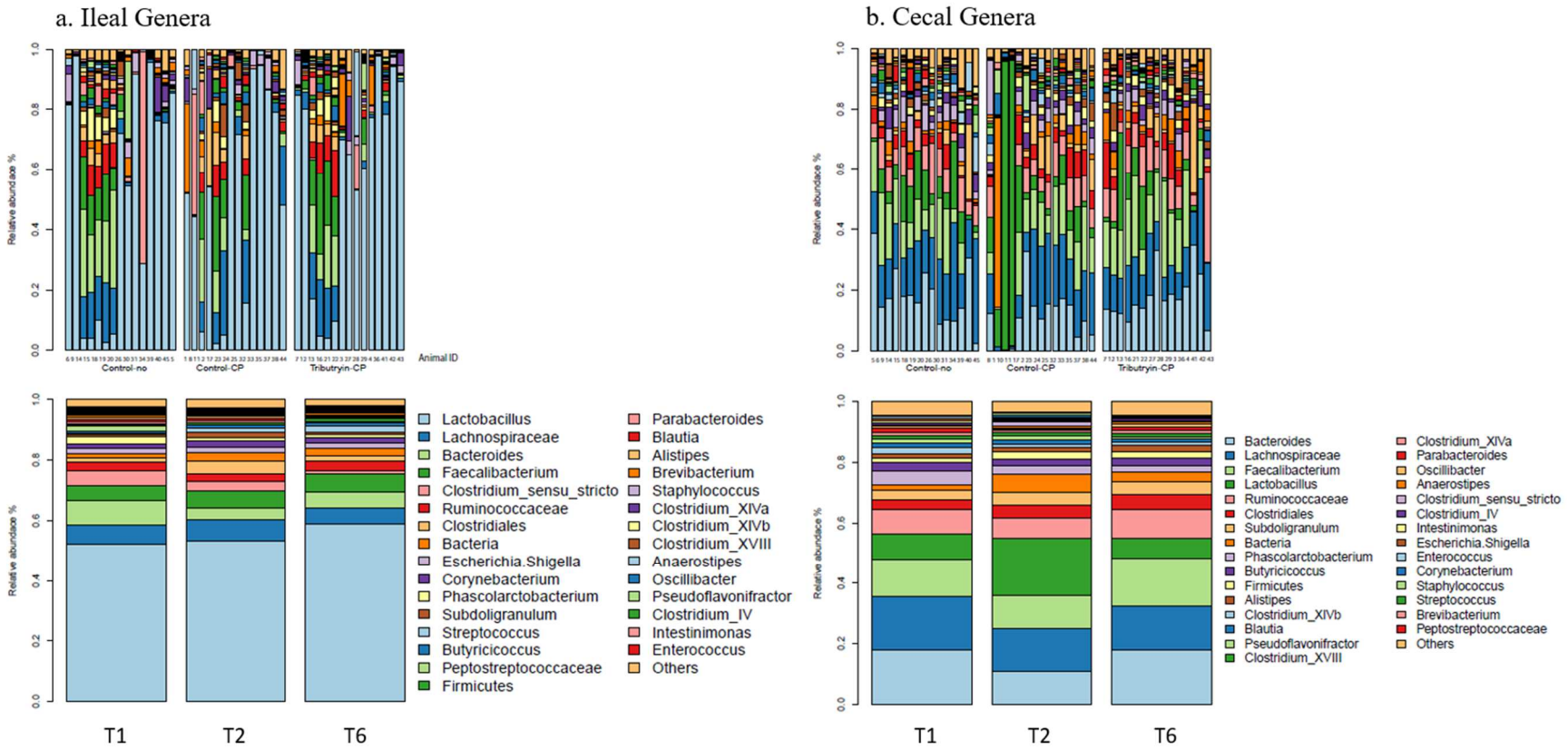

Figure 4. Stacked bar charts of relative abundance of ileal (a) and cecal (b) genera found in the T1 (negative control), T2 (non-medicated, *C. perfringens* and *E. maxima* challenged), and T6 (*C. perfringens* and *E. maxima* challenged, tributyrin supplemented) groups.

3.4 Relative Abundance of Ileal and Cecal Genera

At the genus level, there was no significant difference ($p <= 0.05$, LDA score >2.0) among the three (3) treatment groups of the ileal (Figure 4. a) or cecal (Figure 4. b) segment samples as determined by relative abundance measures at the OTU level. Notably, there was considerable intra-animal variability within each group of the ileal samples. As expected, the cecal samples had an overall greater microbial diversity when compared to the samples taken from the ileum, as typically found in broiler intestinal microbiota research (Pan & Yu, 2013). Within each treatment group of the ileum, there are several samples that more closely mimic the microbial community found in our cecal samples than they do the expected ileal communities of a broiler chicken. Numerically, the T1 group of the ileal samples exhibited a greater relative abundance of genera *Bacteroides* on average, when compared to the T2 group. In comparison, the T2 positive control group exhibited a greater abundance of *Clostridiales* genera than the other groups within the ileal samples. Another genus of interest in the ileum, *Clostridium sensu stricto,* is highly prevalent in the T1 group, tapers off in abundance in the T2 group, and is out populated in the T6 treatment group.

At the genus level, the relative abundance of cecal samples among treatment groups is not significantly different ($p<0.05$). Minor changes in community membership are compared numerically. The cecal sample replicates within each treatment group appear to be more uniform than the ileal samples when viewed side by side. In all groups of cecal samples, the top four most abundant genera, when combined, account for more than 50 percent of microbial abundance. These genera in descending order are *Bacteroides, Lachnospiriceae, Faecalibacterium,* and *Lactobacillus.* In general, the T2 group within the cecal samples showed decreased relative abundance of *Bacteroides* genera but increased relative abundance of *Lactobacillus* genera

compared to the other treatment groups. Statistically, there was no difference in genera among treatment groups.

3.5 Identification of OTU Enrichment by Random Forest

The negative control samples in the cecum shared an increased abundance of OTU 175 *Clostridium XIVa* (p=0.041), OTU 35 Lachnospiraceae (p=0.043), and OTU 4 *Bacteroides* (p=0.021) with the T6 treatment group according to RF. The DNA sequence corresponding to OTU 4 *Bacteroides* was identified by BLAST as *Phocaeicola dorei* with 100% identification accuracy. In the ileum, the negative control (T1) group shared a decreased abundance of OTU 385 *Lachnospiraceae* (p=0.067) with the T6 treatment group, while the NE challenged group (T2) exhibited enrichment of this OTU. This decreased abundance was not statistically significant. The out-of-bag (OOB) estimate for ileal samples was 90.91% and the OOB for cecal samples was 77.78%.

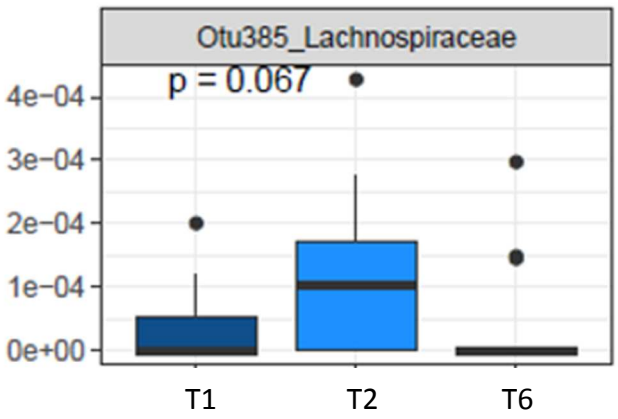

Figure 5. Boxplot of enriched OTUs in the T1 (negative control), T2 (non-medicated, *C. perfringens* and *E. maxima* challenged), and T6 (*C. perfringens* and *E. maxima* challenged, tributyrin supplemented) groups of ileal samples as predicted by Random Forest analysis.

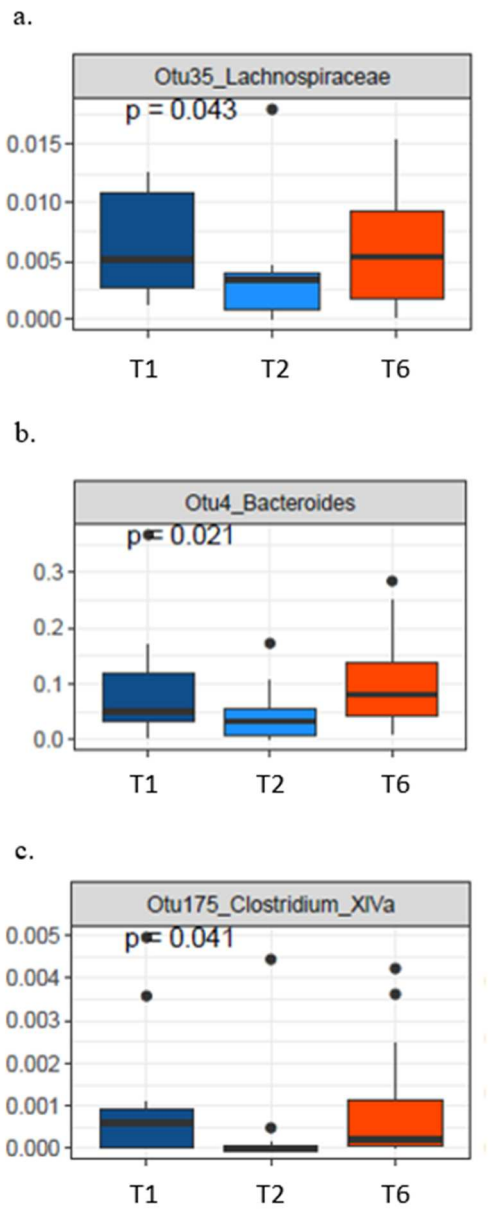

Figure 6. Boxplot of enriched OTUs in the T1 (negative control), T2 (non-medicated, *C. perfringens* and *E. maxima* challenged), and T6 (*C. perfringens* and *E. maxima* challenged, tributyrin supplemented) groups of cecal samples as predicted by Random Forest analysis.

4. Discussion.

4.1 Potential Explanations for Lack of Significant Treatment Effect on *C. perfringens* Proliferation

The community structure and membership of microbial species were statistically indistinguishable among treatment groups and therefore not affected by the NE challenge or the tributyrin treatment according to the presence-absence-based Jaccard dissimilarity index and the abundance-based Bray-Curtis dissimilarity index, which are the gold standard tests of beta diversity in microbial ecology research (Schroeder et al., 2018). As a result, the relative abundance of *C. perfringens,* the causative agent of NE in chickens, was not significantly different among the three groups. A possible explanation for the lack of a significant effect on challenged and treated birds (T6 group) is butyrate's inability to directly impact microbial inhibition. It has been reported that butyrate prevents lesions associated with NE in broilers, but no known antimicrobial effect on *C. perfringens* exists directly (Timbermont et al., 2010). Birds in this *in vivo* study were orally challenged with approximately 4×10^8 CFU of *C. perfringens* three times a day on days 18 and 19 and the butyrate treatment consisted of 330g butyric acid/ton and 250g butyric acid/ton for the starter and grower diets, respectively. The necrotic lesion alleviating effect of butyrate is likely due to the enhanced epithelial health and proliferation, increased mucin production, and other host effects (Guilloteau et al., 2010; Timbermont et al., 2010). Our finding of no significant difference in the relative abundance of *C. perfringens* among the three treatment groups while simultaneously exhibiting a reduction in lesion score in our butyrate treatment group may be due to the known effects of butyrate on necrotic lesion reduction without direct inhibition *C. perfringens* colonization. There have been other reports of butyrate-derived treatments successfully inhibiting *C. perfringens* colonization, however. In an

in vitro setting, Namkung, et al., 2011 found that butyric acid derivatives, mono-, di-, and tri-butyrin, successfully inhibited *C. perfringens* growth, especially at low levels of aerobic inoculation (10^5 CFU *C. perfringens*). The inhibitory effects of the butyrate derivatives were less pronounced when a higher initial inoculation of *C.* perfringens (10^7 CFU) and anaerobic methods were used (Namkung, et al., 2011). When broiler cecal contents were also inoculated onto the culture media, however, a significant inhibitory effect of *C. perfringens* using the 50% monobutyrin treatment was observed with little dose effect (Namkung et al., 2011). It should also be mentioned that in that study tributyrin was not tested alone for *C. perfringens* inhibition and when it was used in combination with other butyrate derivatives, a monobutyrin treatment still outperformed tributyrin containing treatments (Namkung, et al., 2011). A stark difference between our work and these studies, neither Timbermont (2010) nor Namkung (2011) used 16S rRNA gene sequencing techniques to evaluate the approximate *C. perfringens* populations.

The finding of no inhibitory effect through butyrate supplementation can be partially supported through the literature, but surprisingly, our challenged and untreated birds (T2 group) did not exhibit a significant shift in microbial composition, particularly in the relative abundance of *C. perfringens.* The relationship between *C. perfringens* challenge, NE, and microbial dysbiosis is complex. Multiple predisposing factors in various combinations are commonly included in trials for induction of NE including Coccidia challenge, high-protein fishmeal diets, and inoculation with *C. perfringens* (Shojadoost et al., 2012). In previous work, birds challenged with *C. perfringens* and fed a high-protein fishmeal-based diet displayed a significant shift in the microbial composition within the ceca determined by 16S rRNA gene sequencing (Stanley et al., 2012). This shift included a displacement of *Lactobacillus johnsonii, Lactobacillus ferementum, Weisella confusa,* and numerous butyrate-producing bacteria in favor of *Lactobacillus crispatus,*

Lactobacillus pontis, Lactobacillus ultunese, *Lactobacillus salivarius, Ruminoccocus albus,* and *C. perfringens* (Stanley et al., 2012). Also using 16S rRNA gene sequencing, it has been found that the ceca of birds challenged with a high-protein fishmeal diet exhibit a significant reduction in *Lactobacillus johnsonii* and *Lactobacillus acidophilus,* as well as species belonging to the *Lachnospiraceae* family, and increase in *Lactobacillus reuteri*, and *Lactobacillus animalis* (Wu et al., 2014). Again, 16S rRNA sequencing revealed that birds challenged with *C. perfringens,* coccidia, and a fishmeal diet exhibited an increase in abundance of *Lactobacillus* and *Dorea* species in the cecum (Lin, Y. et al., 2017). Based on these reports, it is surprising that our *C. perfringens* challenge group did not exhibit a significantly different microbial composition compared to our negative control, especially in the ceca samples. The possible explanations for this include the *C. perfringens* vector, the inability of butyrate to directly affect the microbiota, or that the NE disease phase had passed by day 21. Other potential explanations including sequencing procedure and choice of hypervariable DNA region primer, though plausible, seem less likely to have a damaging impact on the results of this study.

It is widely accepted that a 15-hour culture grown on fluid thioglycolate medium (with dextrose) (FTG) is acceptable for inducing a severe NE challenge in broilers (Shojadoost et al., 2012). This culture technique will result in 10^7-10^9 CFU *C. perfringens*/mL, which is the normal amount accepted for a successful challenge in broilers (Shojadoost et al., 2012). The culture (CP6) used in our study exactly matches these recommendations for the successful induction of NE in broilers. We can conclude that the *C. perfringens* culture (CP6) was suitable and thus not responsible for the lack of microbial diversity among the treatment groups. A possible error in the trial design was the vector of transmission. In the literature, broilers are inoculated with *C. perfringens* successfully through the inclusion of the culture in the diet and fed ad libitum for 3-5

consecutive days up until euthanasia (Shojadoost et al., 2012). Our study utilized a water vector for *C. perfringens* transmission and the challenge only took place for two consecutive days before target mortality was reached and the trial was terminated. In the literature, it is less common for birds to be inoculated with *C. perfringens* through the water source, but when Timbermont (2010) did so, birds were challenged with approximately 4×10^8 CFU three times a day for four consecutive days before euthanasia. A critique of our study was the choice of water vector over diet vector, the limited exposure time of 30 minutes once daily, and the limited number of challenge days.

The disease NE is not entirely understood and there are possibilities relating to the nature of the disease itself that could have led to our findings. NE causes severe gastrointestinal distress and death in an acute event and milder symptoms, if any, in a chronic form. It has been reported that in an acute setting, chickens have died within thirty minutes after symptoms were first observed, and most deaths occurred between 36 and 48 hours after initial exposure to *C. perfringens* contaminated feed (Long et al., 1976). It was also reported in that study that the birds that survived the NE disease challenge had normal intestinal findings upon gross examination. In our study, birds that died as a result of the challenge were not sampled and those that survived may have recovered before the samples were taken. Birds in our study were also sampled on day 21, approximately 72 hours after their first exposure to *C. perfringens.*

Microbiota research is advanced, widespread, and has grown at an unprecedented rate with the advent of Next-Generation Sequencing techniques. The current microbiome epoch is inevitably accompanied by challenges. There is not to date an established universal protocol for DNA extraction, sequencing, and analysis to outline the diversity and richness within the microbiome of a given subject and among others. Variation in results due to the use of different sequencing

platforms and bioinformatics pipelines (Allali et al., 2017), alternate sample types (Knudsen et al., 2016), and DNA isolation procedures (Fiedorová et al., 2019; Knudsen et al., 2016) provokes concern over reproducibility and reliability of findings obtained in microbiome research. Further complicating the scientific inferences are issues involving the disparity in the level of sequencing depth and coverage (Zaheer et al., 2018), as well as a selection of the target gene region (Rintala et al., 2017). We dove into these and other possibilities as an explanation for our findings.

The first step in 16S rRNA gene sequencing is DNA extraction from the intestinal sample. Great care must be taken when handling samples with low microbial DNA contents, such as tissue samples, to prevent contamination and decrease the potential for false-positive results (Greathouse et al., 2018). Luckily, the ileum and cecum are areas known to harbor robust microbial communities, and as such, are less susceptible to bias and contamination during the extraction process, but not entirely exempt (Greathouse et al., 2018). Commercially, there are several DNA extraction kits available to researchers that are suitable for use with chicken intestinal content and fecal samples. In one of few studies comparing the quality of the commercially available DNA extraction kits for their use with fecal samples in terms of DNA quality and quantity, performance extracting Gram-positive bacteria, accuracy, and repeatability, the DNeasy PowerLyzer® PowerSoil® DNA Isolation Kit performed best overall, although no kit was able to perform the best in all categories (Elie et al., 2020). Furthermore, this extraction method has been used to characterize the chicken intestinal microbial community in the literature previously (Glendinning et al., 2019). The quality of the DNA extract is extremely important to downstream sequencing integrity and reliability, and the literature supports our use of the DNeasy PowerLyzer® PowerSoil® DNA Isolation Kit. There are additional steps and protocols to be considered and filtered for reliable 16S rRNA gene sequencing.

It is widely accepted that the target hypervariable region of the 16S rRNA gene has a great impact on gut microbiota analysis (Rintala et al., 2017; Johnson et al., 2019; Darwish et al., 2021). Most researchers agree that the combination of V1-V4 regions is reliably accurate and suitable for meaningful bacterial classification within the chicken gut, though no universal mandatory standard currently exists (Kim, et al., 2011; Di Bella et al., 2013; Darwish et al., 2021). It has been found that targeting the V4 region delivers microbial diversity results comparable with those of full-length 16S gene sequencing, called shotgun sequencing (Ballou et al., 2016; Darwish et al., 2021). Several comparisons of the sequencing platforms available have found that Illumina HiSeq, and for smaller projects, the MiSeq systems are highly reliable for 16S rRNA microbial research and heavily utilized in the research community (Caporaso et al., 2012; Tremblay et al., 2015). Separately, the bioinformatics pipelines *Mothur* and *Qiime* stand out in the literature as the most outstanding software packages and are frequently chosen for chicken GIT microbiota research, increasing the comparison value of our study to the literature (Nilakanta et al., 2014). Based on this information, the gene sequencing and data analysis procedures chosen for this study are in line with the most up-to-date standard represented in the literature of GIT microbiota research in chickens.

4.2 Random Forest Identification of Enriched Features

There is a possibility that some of the samples used in this study were mislabeled, which may have led to the relative abundance of our ileal samples being inconsistent with what has been reported in the literature. Although the Bray-Curtis and Jaccard analyses revealed no significant treatment effect, the decision tree algorithm, Random Forest, was able to identify a few enriched features in the negative control (T1) and butyrate treatment group (T6) as distinct from the challenge group (T2). The reliability of the random forest predictions relies on the knowledge

that RF can maintain a consistent prediction accuracy until 30-40% of the total samples are mislabeled, as determined by Knights (2011). In this longitudinal 16S rRNA sequencing study, several “late” samples were switched to “early”, similar to our potential mislabeling of distinctive “cecum” samples as “ileum”. Additionally, based on observed relative abundance differences of the ileal samples where approximately less than half of each sample is dominated by *Lactobacillus*, 5 samples from the T1 group, 5 samples from the T2 group, and 4 samples from the T6 group appear to be mislabeled. This results in 14 of the total 45 ileal samples as potentially mislabeled, or an error of 31.1%, which falls within the range for maintenance of an accurate estimate of RF (Knights et al., 2011). Although this is not an ideal scenario, this may mean some inference obtained by RF can be helpful in explaining the relationship between NE, the tributyrin treatment, and the associated microbial modulation of each.

The classification algorithm, Random Forest (RF), predicted a few features that could possibly represent a link between the tributyrin treatment and the normalization of the gut microbiota after NE infection. In the cecum, RF identified an increase in abundance of OTU 175 *Clostridium* XIVa, OTU35 *Lachnospiraceae*, and OTU4 *Bacteroides* in the negative control and butyrate treatment group compared to the *C. perfringens* challenge group. Bacteria belonging to the *Clostridium* XIVa cluster, also known as the *Clostridium Coccoides* group, are known to be lactate-utilizing and butyrate-producing. The favorable functions of butyrate in the GIT are diverse and well-reviewed. Butyrate is a major energy source to intestinal epithelia, regulates cell proliferation and contributes to programmed cell death, enhances intestinal barrier function, and alleviates local inflammation (Guilloteau et al., 2010). Butyrate strengthens the gut barrier function through the upregulation of tight junctions, limiting barrier permeability to pathogen invasion and circulation (Chen et al., 2020). Our work corroborated previous work showing that

members of the *Clostridium* cluster XIVa are commonly found in the cecum of healthy broilers (Zhu et al., 2002; Guilloteau et al., 2010; Ijaz et al., 2018). Furthermore, it has been demonstrated that organic acid-based feed supplements increase the level of *C. Coccoides* cluster species in the cecum of healthy broiler chickens (Palamidi et al., 2018). The RF prediction of cluster XIVa Clostridia as prevalent in the cecum of negative control and butyrate treatment group birds is supported in the literature.

The BLAST analysis identified OTU 35 *Lachnospiraceae* as *Lacrimispora indolis* with 96% identification accuracy. It can be assumed that this OTU is some relative of *Lacrimispora indolis* because the identity threshold is below 97% (Reller et al., 2007). Previously named *Clostridium indolis*, this member of the *Lachnospiraceae* family possesses genes associated with lactate, citrate, malate, and succinate utilization as well as nitrogen fixation (Haas et al., 2020; Biddle et al., 2014). This bacterium is not well understood in terms of physiology and there are contradicting reports regarding the characterization of its functions in lactate or other carbohydrate utilization (Biddle et al., 2014). It has even been isolated alongside *C. perfringens* in human patients suffering with clostridium pseudobacteraemia. It is puzzling to observe an enrichment in this OTU outside of the *Clostridium perfringens* challenge group (T2).

The final significant enriched OTU of the cecal samples as determined by RF, OTU 4 *Bacteroides* was determined by BLAST analysis to be *Phocaeicola dorei* with 100% identification accuracy. There is a strong association between *Phocaeicola* spp. found in the gut and host health. In humans, bacteria belonging to this genus are known for enhancing immunity and enteric pathogen control (Wang et al., 2021). *Phocaeicola dorei* is closely related to *Phocaeicola vulgatus,* previously known as *Bacteroides vulgatus,* whose role in intestinal health is contradictory (Cobo et al., 2022; Lück et al., 2022). *Phocaeicola vulgatus* is highly abundant

in the human GIT and it and other *Phocaeicola* spp. are excellent SCFA producers (Lück et al., 2022), but there are reported associations of *Phocaeicola dorei* with human disease (Usyk et al., 2021; Cobo et al., 2022). In neonates, an increased abundance of both *C. perfringens* and *P. dorei* is associated with necrotizing enterocolitis, the human equivalent of NE in chickens (Heida et al., 2016). Our finding of enrichment of *P. dorei* in the T1 and T6 groups, rather than the T2 group, is incredibly surprising based on the relationship of these microbiota in the literature.

In the ileum, the abundance of OTU 385 *Lachnospiraceae*, was greatest in the *C. perfringens* challenge group, but less abundant in the negative control and the butyrate treatment groups. This OTU was identified by BLAST analysis as *Christensenella minuta* with 86.56% identity. Most taxonomists accept a percent identity score of ≥97% to classify a microorganism to a genus and ≥99% to a species using 16S rRNA gene sequence alignment (Reller et al., 2007). Based on this requirement, it is likely that OTU 385 is a novel relative of *C. minuta. Christensenella minuta* itself is a newly discovered strictly anaerobic, non-spore-forming, Gram-negative, rod-shaped bacterium isolated from the GIT of adult humans (Morotomi et al., 2012). This member of the *Christensenellaceae* family within the order *Clostridiales,* is associated with weight loss in humans and chickens and is being considered as a potential enteric pathogen (Goodrich et al., 2014; Borelli et al., 2017; Yang, et al., 2018). More work is needed to determine the functional role of this novel bacterium in the intestinal microbial community of humans and chickens, and whether this bacterium has any relationship with *C. perfringens* proliferation.

LITERATURE CITED

Allaband, C., McDonald, D., Vázquez-Baeza, Y., Minich, J. J., Tripathi, A., Brenner, D. A., ... & Knight, R. (2019). Microbiome 101: studying, analyzing, and interpreting gut microbiome data for clinicians. Clinical gastroenterology and hepatology, 17(2), 218-230.

Allali, I., Arnold, J. W., Roach, J., Cadenas, M. B., Butz, N., Hassan, H. M., ... & Azcarate-Peril, M. A. (2017). A comparison of sequencing platforms and bioinformatics pipelines for compositional analysis of the gut microbiome. BMC microbiology, 17(1), 1-16.

Ballou, A. L., Ali, R. A., Mendoza, M. A., Ellis, J. C., Hassan, H. M., Croom, W. J., & Koci, M. D. (2016). Development of the chick microbiome: how early exposure influences future microbial diversity. Frontiers in veterinary science, 3, 2.

Biddle, A. S., Leschine, S., Huntemann, M., Han, J., Chen, A., Kyrpides, N., ... & Blanchard, J. L. (2014). The complete genome sequence of Clostridium indolis DSM 755 T. Standards in genomic sciences, 9(3), 1089-1104.

Borrelli, L., Coretti, L., Dipineto, L., Bovera, F., Menna, F., Chiariotti, L., ... & Fioretti, A. (2017). Insect-based diet, a promising nutritional source, modulates gut microbiota composition and SCFAs production in laying hens. Scientific reports, 7(1), 1-11.

Bortoluzzi, C., Vieira, B. S., Hofacre, C., & Applegate, T. J. (2019). Effect of different challenge models to induce necrotic enteritis on the growth performance and intestinal microbiota of broiler chickens. Poultry science, 98(7), 2800-2812.

C. F. Hofacre, personal communication, July 7, 2020 – November 22, 2021

Caly, D. L., D'Inca, R., Auclair, E., & Drider, D. (2015). Alternatives to antibiotics to prevent necrotic enteritis in broiler chickens: a microbiologist's perspective. Frontiers in microbiology, 6, 1336.

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Huntley, J., Fierer, N., ... & Knight, R. (2012). Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. The ISME journal, 6(8), 1621-1624.

Chen, W., Zhang, S., Wu, J., Ye, T., Wang, S., Wang, P., & Xing, D. (2020). Butyrate-producing bacteria and the gut-heart axis in atherosclerosis. Clinica Chimica Acta, 507, 236-241.

Cobo, F., Pérez-Carrasco, V., Rodríguez-Guerrero, E., Sampedro, A., Rodríguez-Granger, J., García-Salcedo, J. A., & Navarro-Marí, J. M. (2022). Misidentification of Phocaeicola (Bacteroides) dorei in two patients with bacteremia. Anaerobe, 102544.

Darwish, N., Shao, J., Schreier, L. L., & Proszkowiec-Weglarz, M. (2021). Choice of 16S ribosomal RNA primers affects the microbiome analysis in chicken ceca. Scientific Reports, 11(1), 1-15.

Di Bella, J. M., Bao, Y., Gloor, G. B., Burton, J. P., & Reid, G. (2013). High throughput sequencing methods and analysis for microbiome research. Journal of microbiological methods, 95(3), 401-414.

Dumonceaux, T. J., Hill, J. E., Hemmingsen, S. M., & Van Kessel, A. G. (2006). Characterization of intestinal microbiota and response to dietary virginiamycin supplementation in the broiler chicken. Applied and environmental microbiology, 72(4), 2815-2823.

Elie, C., Perret, M., Louis, K., Fritah-Lafont, A., Leissner, P., Vachon, C., ... & Saliou, A. (2020). A unique and reliable fecal DNA extraction method for 16S rRNA gene and shotgun metagenomic sequencing in the analysis of the human gut microbiome.

Fasina, Y. O., Newman, M. M., Stough, J. M., & Liles, M. R. (2016). Effect of Clostridium perfringens infection and antibiotic administration on microbiota in the small intestine of broiler chickens. Poultry science, 95(2), 247-260.

Fiedorová, K., Radvanský, M., Němcová, E., Grombiříková, H., Bosák, J., Černochová, M., ... & Freiberger, T. (2019). The impact of DNA extraction methods on stool bacterial and fungal microbiota community recovery. Frontiers in microbiology, 10, 821.

George, B. A., Quarles, C. L., & Fagerberg, D. J. (1982). Virginiamycin effects on controlling necrotic enteritis infection in chickens. Poultry Science, 61(3), 447-450.

Gerber, P., Opio, C., & Steinfeld, H. (2007). Poultry production and the environment—a review. Animal production and health division, Food and Agriculture Organization of the United Nations, Viale delle Terme di Caracalla, 153.

Glendinning, L., Watson, K. A., & Watson, M. (2019). Development of the duodenal, ileal, jejunal and caecal microbiota in chickens. Animal microbiome, 1(1), 1-11.

Goodrich, J. K., Waters, J. L., Poole, A. C., Sutter, J. L., Koren, O., Blekhman, R., ... & Ley, R. E. (2014). Human genetics shape the gut microbiome. Cell, 159(4), 789-799.

Greathouse, K. L., White, J. R., Vargas, A. J., Bliskovsky, V. V., Beck, J. A., von Muhlinen, N., ... & Harris, C. C. (2018). Interaction between the microbiome and TP53 in human lung cancer. Genome biology, 19(1), 1-16.

Guilloteau, P., Martin, L., Eeckhaut, V., Ducatelle, R., Zabielski, R., & Van Immerseel, F. (2010). From the gut to the peripheral tissues: the multiple effects of butyrate. Nutrition research reviews, 23(2), 366-384.

Haas, K. N., & Blanchard, J. L. (2020). Reclassification of the Clostridium clostridioforme and Clostridium sphenoides clades as Enterocloster gen. nov. and Lacrimispora gen. nov., including reclassification of 15 taxa. International Journal of Systematic and Evolutionary Microbiology, 70(1), 23-34.

Heather, J. M., & Chain, B. (2016). The sequence of sequencers: The history of sequencing DNA. Genomics, 107(1), 1-8.

Heida, F. H., van Zoonen, A. G., Hulscher, J. B., Te Kiefte, B. J., Wessels, R., Kooi, E. M., ... & de Goffau, M. C. (2016). A necrotizing enterocolitis-associated gut microbiota is present in the meconium: results of a prospective study. Clinical Infectious Diseases, 62(7), 863-870.

Hofacre, C. L., Mathis, G. F., Lumpkins, B. S., Sygall, R., Vaessen, S., Hofacre, C. S., ... & Clanton, E. (2020). Efficacy of Butyric and Valeric Acid Esters in a Necrotic Enteritis Challenge Model. Avian Diseases, 64(3), 407-414.

Hofacre, C. L., Smith, J. A., & Mathis, G. F. (2018). An optimist's view on limiting necrotic enteritis and maintaining broiler gut health and performance in today's marketing, food safety, and regulatory climate. Poultry science, 97(6), 1929-1933.

Huyghebaert, G., Ducatelle, R., & Van Immerseel, F. (2011). An update on alternatives to antimicrobial growth promoters for broilers. The Veterinary Journal, 187(2), 182-188.

Ijaz, U. Z., Sivaloganathan, L., McKenna, A., Richmond, A., Kelly, C., Linton, M., ... & Gundogdu, O. (2018). Comprehensive longitudinal microbiome analysis of the chicken cecum reveals a shift from competitive to environmental drivers and a window of opportunity for Campylobacter. Frontiers in microbiology, 2452.

Immerseel, F. V., Buck, J. D., Pasmans, F., Huyghebaert, G., Haesebrouck, F., & Ducatelle, R. (2004). Clostridium perfringens in poultry: an emerging threat for animal and public health. Avian pathology, 33(6), 537-549.

Johnson, J. S., Spakowicz, D. J., Hong, B. Y., Petersen, L. M., Demkowicz, P., Chen, L., ... & Weinstock, G. M. (2019). Evaluation of 16S rRNA gene sequencing for species and strain-level microbiome analysis. Nature communications, 10(1), 1-11.

Kim, M., Morrison, M., & Yu, Z. (2011). Evaluation of different partial 16S rRNA gene sequence regions for phylogenetic analysis of microbiomes. Journal of microbiological methods, 84(1), 81-87

Knights, D., Kuczynski, J., Koren, O., Ley, R. E., Field, D., Knight, R., ... & Kelley, S. T. (2011). Supervised classification of microbiota mitigates mislabeling errors. The ISME journal, 5(4), 570-573.

Knudsen, B. E., Bergmark, L., Munk, P., Lukjancenko, O., Priemé, A., Aarestrup, F. M., & Pamp, S. J. (2016). Impact of sample type and DNA isolation procedure on genomic inference of microbiome composition. MSystems, 1(5), e00095-16.

Kozich, J. J., Westcott, S. L., Baxter, N. T., Highlander, S. K., & Schloss, P. D. (2013). Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. Applied and environmental microbiology, 79(17), 5112-5120.

Latorre, J. D., Adhikari, B., Park, S. H., Teague, K. D., Graham, L. E., Mahaffey, B. D., ... & Tellez, G. (2018). Evaluation of the epithelial barrier function and ileal microbiome in an

established necrotic enteritis challenge model in broiler chickens. Frontiers in veterinary science, 199.

Li, J., Hou, Y., Yi, D., Zhang, J., Wang, L., Qiu, H., ... & Gong, J. (2015). Effects of tributyrin on intestinal energy status, antioxidative capacity and immune response to lipopolysaccharide challenge in broilers. Asian-Australasian Journal of Animal Sciences, 28(12), 1784.

Lin, Y., Xu, S., Zeng, D., Ni, X., Zhou, M., Zeng, Y., ... & Li, G. (2017). Disruption in the cecal microbiota of chickens challenged with Clostridium perfringens and other factors was alleviated by Bacillus licheniformis supplementation. PloS one, 12(8), e0182426.

Long, J. R., & Truscott, R. B. (1976). Necrotic enteritis in broiler chickens. III. Reproduction of the disease. Canadian Journal of Comparative Medicine, 40(1), 53.

Lu, J., Idris, U., Harmon, B., Hofacre, C., Maurer, J. J., & Lee, M. D. (2003). Diversity and succession of the intestinal bacterial community of the maturing broiler chicken. Applied and environmental microbiology, 69(11), 6816-6824.

Lück, R., & Deppenmeier, U. (2022). Genetic tools for the redirection of the central carbon flow towards the production of lactate in the human gut bacterium Phocaeicola (Bacteroides) vulgatus. Applied Microbiology and Biotechnology, 1-15.

Mohd Shaufi, M. A., Sieo, C. C., Chong, C. W., Gan, H. M., & Ho, Y. W. (2015). Deciphering chicken gut microbial dynamics based on high-throughput 16S rRNA metagenomics analyses. Gut pathogens, 7(1), 1-12.

Moquet, P. C. A., Onrust, L., Van Immerseel, F., Ducatelle, R., Hendriks, W. H., & Kwakkel, R. P. (2016). Importance of release location on the mode of action of butyrate derivatives in the avian gastrointestinal tract. World's Poultry Science Journal, 72(1), 61-80.

Morotomi, M., Nagai, F., & Watanabe, Y. (2012). Description of Christensenella minuta gen. nov., sp. nov., isolated from human faeces, which forms a distinct branch in the order Clostridiales, and proposal of Christensenellaceae fam. nov. International journal of systematic and evolutionary microbiology, 62(1), 144-149.

M'Sadeq, S. A., Wu, S., Swick, R. A., & Choct, M. (2015). Towards the control of necrotic enteritis in broiler chickens with in-feed antibiotics phasing-out worldwide. Animal Nutrition, 1(1), 1-11.

Namkung, H., Yu, H., Gong, J., & Leeson, S. (2011). Antimicrobial activity of butyrate glycerides toward Salmonella Typhimurium and Clostridium perfringens. Poultry Science, 90(10), 2217-2222.

National Research Council. (2010). Guide for the care and use of laboratory animals.

National Research Council. (1994). Nutrient Requirements of Poultry: Ninth Revised Edition, 1994. Washington, DC: The National Academies Press.

Nilakanta, H., Drews, K. L., Firrell, S., Foulkes, M. A., & Jablonski, K. A. (2014). A review of software for analyzing molecular sequences. BMC Research Notes, 7(1), 1-9.

Palamidi, I., & Mountzouris, K. C. (2018). Diet supplementation with an organic acids-based formulation affects gut microbiota and expression of gut barrier genes in broilers. Animal Nutrition, 4(4), 367-377.

Pan, D., & Yu, Z. (2014). Intestinal microbiome of poultry and its interaction with host and diet. Gut microbes, 5(1), 108-119.

Putman, B., Thoma, G., Burek, J., & Matlock, M. (2017). A retrospective analysis of the United States poultry industry: 1965 compared with 2010. Agricultural Systems, 157, 107-117.

Reller, L. B., Weinstein, M. P., & Petti, C. A. (2007). Detection and identification of microorganisms by gene amplification and sequencing. Clinical infectious diseases, 44(8), 1108-1114.

Rintala, A., Pietilä, S., Munukka, E., Eerola, E., Pursiheimo, J., Laiho, A., . . . Huovinen, P. (2017). Gut Microbiota Analysis Results Are Highly Dependent on the 16S rRNA Gene Target Region, Whereas the Impact of DNA Extraction Is Minor. Journal of Biomolecular Techniques : JBT, 28(1), 19-30. doi:10.7171/jbt.17-2801-003

Schroeder, P. J., & Jenkins, D. G. (2018). How robust are popular beta diversity indices to sampling error?. Ecosphere, 9(2), e02100.

Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W. S., & Huttenhower, C. (2011). Metagenomic biomarker discovery and explanation. Genome biology, 12(6), 1-18.

Shojadoost, B., Vince, A. R., & Prescott, J. F. (2012). The successful experimental induction of necrotic enteritis in chickens by Clostridium perfringens: a critical review. Veterinary research, 43(1), 1-12.

Stanley, D., Hughes, R. J., & Moore, R. J. (2014). Microbiota of the chicken gastrointestinal tract: influence on health, productivity and disease. Applied microbiology and biotechnology, 98(10), 4301-4310.

Stanley, D., Keyburn, A. L., Denman, S. E., & Moore, R. J. (2012). Changes in the caecal microflora of chickens following Clostridium perfringens challenge to induce necrotic enteritis. Veterinary microbiology, 159(1-2), 155-162.

Timbermont, L., Lanckriet, A., Dewulf, J., Nollet, N., Schwarzer, K., Haesebrouck, F., ... & Van Immerseel, F. (2010). Control of Clostridium perfringens-induced necrotic enteritis in broilers by target-released butyric acid, fatty acids and essential oils. Avian Pathology, 39(2), 117-121.

Tremblay, J., Singh, K., Fern, A., Kirton, E. S., He, S., Woyke, T., ... & Tringe, S. G. (2015). Primer and platform effects on 16S rRNA tag sequencing. Frontiers in microbiology, 6, 771.

Usyk, M., Pandey, A., Hayes, R. B., Moran, U., Pavlick, A., Osman, I., ... & Ahn, J. (2021). Bacteroides vulgatus and Bacteroides dorei predict immune-related adverse events in immune checkpoint blockade treatment of metastatic melanoma. Genome medicine, 13(1), 1-11.

Wang, C., Li, S., Zhang, Z., Yu, Z., Yu, L., Tian, F., ... & Zhai, Q. (2021). Phocaeicola faecalis sp. nov., a strictly anaerobic bacterial strain adapted to the human gut ecosystem. Antonie van Leeuwenhoek, 114(8), 1225-1235.

Wei, S., Morrison, M., & Yu, Z. (2013). Bacterial census of poultry intestinal microbiome. Poultry science, 92(3), 671-683.

Wei, X., Tsai, T., Knapp, J., Bottoms, K., Deng, F., Story, R., ... & Zhao, J. (2020). ZnO modulates swine gut microbiota and improves growth performance of nursery pigs when combined with peptide cocktail. Microorganisms, 8(2), 146.

Williams, R. B. (2005). Intercurrent coccidiosis and necrotic enteritis of chickens: rational, integrated disease management by maintenance of gut integrity. Avian pathology, 34(3), 159-180.

Wu, S. B., Rodgers, N., & Choct, M. (2010). Optimized necrotic enteritis model producing clinical and subclinical infection of Clostridium perfringens in broiler chickens. Avian diseases, 54(3), 1058-1065.

Wu, S. B., Stanley, D., Rodgers, N., Swick, R. A., & Moore, R. J. (2014). Two necrotic enteritis predisposing factors, dietary fishmeal and Eimeria infection, induce large changes in the caecal microbiota of broiler chickens. Veterinary microbiology, 169(3-4), 188-197.

Xiao, Y., Xiang, Y., Zhou, W., Chen, J., Li, K., & Yang, H. (2017). Microbial community mapping in intestinal tract of broiler chicken. Poultry science, 96(5), 1387-1393.

Yang, Y., Gu, H., Sun, Q., & Wang, J. (2018). Effects of Christensenella minuta lipopolysaccharide on RAW 264.7 macrophages activation. Microbial pathogenesis, 125, 411-417.

Zaheer, R., Noyes, N., Ortega Polo, R., Cook, S. R., Marinier, E., Van Domselaar, G., ... & McAllister, T. A. (2018). Impact of sequencing depth on the characterization of the microbiome and resistome. Scientific reports, 8(1), 1-11.

Zhu, X. Y., Zhong, T., Pandya, Y., & Joerger, R. D. (2002). 16S rRNA-based analysis of microbiota from the cecum of broiler chickens. Applied and environmental microbiology, 68(1), 124-137.

CHAPTER III.

Conclusion

1. Conclusion

The purpose of this research was to determine a) the validity of microbial analysis as a diagnostic tool for NE in chickens by highlighting specific microbes that appear to have a significant relationship with the occurrence of this disease and b) the efficacy of using a butyrate-derived supplement as a treatment for NE by highlighting specific microbes that appear to have a relationship with the presence of this supplement and a reduction in NE symptoms. Unfortunately, the *C. perfringens* challenge did not modulate the intestinal microbiota in birds compared to the negative control, and likewise, no significant changes were observed between intestinal microbial populations in the butyrate-treated, *C. perfringens* challenged group compared to the negative control. The random forest analysis determined there were a few enriched microbiota in each treatment group, which may play some role in the development of NE or are affected by the addition of a butyrate supplement. Additionally, the procedures utilized by our lab are comparable, and in some cases, exceed the quality and accuracy of common methods used for DNA sequencing and 16S rRNA microbial data analysis in the scientific community as of 2022. Because of this knowledge, I find it unlikely that our evaluation methods were unable to detect a significant modulation of the intestinal microbial communities. Rather, it seems likely that the inoculation of *C. perfringens* in our challenge and challenge-treatment groups was ineffective, or the butyrate supplement was not responsible for the observed alleviation of symptoms in a way that also altered the intestinal microbiota or both. It is still not well understood how or if butyrate can treat NE in broiler chickens.

Regarding the enriched features determined by Random Forest, more work should be done to focus specifically on the relationship between *C. perfringens* and the presence of bacteria ongoing to the *Clostridium* XIVa cluster. Because the out-of-bag error rate was regrettably high,

a strong association cannot be confidently made based on the results of our study, but the literature supports a negative correlation between *C. perfringens* proliferation and a reduction in LAB in the intestine. Additionally, the increase in abundance of OTU 35 and OTU 6 in the T1 and T6 treatment groups is puzzling. The reliability of the RF results remains in question.

After evaluation of the procedures performed in this study, it is unlikely that any major errors were involved in the DNA extraction, sequencing, or data analysis steps of this study. It is, however, likely that a butyrate-based treatment is less suitable for the treatment of NE in chickens, based on our results and the available literature. There are other methods of disease control, such as probiotic supplementation and *Clostridium perfringens* vaccination, as described in the literature review chapter, that may be more suitable for the treatment of NE. Furthermore, the predisposing factors largely linked to bird management seem to point to a route of prevention through modification of the bird environment, making treatment elucidation a secondary concern. Furthermore, future work in this area should consider alternative methods of NE challenge induction, including another route of *C. perfringens* inoculation and the addition of two or more predisposing factors (i.e., fishmeal diet, coccidia challenge, increased stocking density, etc.). Overall, more work is needed to determine the relationship between butyrate supplementation and intestinal microbiota as well as the efficacy of butyrate as a treatment for NE in chickens.