IMMUNOMETABOLIC SIGNAL TRANSDUCTION PROFILING OF THE CECUM IN CHICKENS FOLLOWING CHALLENGE WITH FUNCTIONAL GUT HEALTH MODIFIERS

A Dissertation

by

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ABSTRACT

Although improved control measures have been implemented, *Salmonella enterica* Enteritidis (*S.* Enteritidis) continues to present an issue every year in US poultry industry. One of the main reasons for this may be the ability of *Salmonella* to survive the initial host immune response and persist. While it is common knowledge that birds infected with *S.* Enteritidis do not manifest any clinical disease symptoms, the mechanisms of persistence occurring in the host during infection have not been widely characterized. The host-pathogen interactions are incredibly complex which makes it difficult to elucidate immune responses and virulence mechanisms. Recent findings in our group have speculated a phenotypic alteration occurring in chicken ceca: early (4 to 48 h) and late (4 to 14 d) infections with *S.* Enteritidis with three distinct immunometabolic phases post-infection: disease resistance, disease tolerance, and homeostasis. This phenomenon may attribute to the disease resistance in the host. Without resolving chronic colonization by *Salmonella*, this results in contaminated poultry products to the public. Consequently, the objective was to elucidate the mechanisms of how *S.* Enteritidis affects immunometabolic tissue phenotype change in early broiler growth.

This objective was approached by testing two different gut physiological modulators in post-hatch broiler chicks: an antibiotic growth promoter (AGP) and a phytobiotic feed additive and their role in the affecting the host phenotype. Using this methodology, an immunometabolic cecal phenotype change was detected in *S.*

Enteritidis challenged birds fed with an AGP based on the shifting microbiota composition and pro-inflammatory response. Furthermore, the addition of a phytobiotic also demonstrated immunometabolic cecal alteration based on the phosphorylation data of the identified metabolic and immune pathways involved in host immunity. For the first time, it is shown that a phytobiotic product initially modulates metabolism while also potentially supporting growth and feed efficiency downstream.

DEDICATION

I would like to dedicate this to my mother, who wanted to pursue her own doctoral degree in English Literature. She was promptly told “an educated woman would never find a suitable husband”. Her encouragement over the years to pursue a career for myself has allowed me to go this far and beyond. Thank you for never pushing me to settle, mom!

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NOMENCLATURE

AGP	Antibiotic growth promoters
AMPK	5' adenosine monophosphate-activated protein kinase
ANOSIM	Analysis of similarities
ANOVA	Analysis of variance
ATA	Alternative to antibiotics
BMD	Bacitracin methylene disalicylate
BP	Biological process
ChT	Chestnut tannins
EDTA	Ethylenediaminetetraacetic acid
FDR	False discovery rate
GIT	Gastrointestinal tract
GM-CSF	Granulocyte macrophage colony-stimulating factor
GO	Gene Ontology
IFN	Interferon
IL-[#]	Interleukin-[number]
KEGG	Kyoto Encyclopedia of Genes and Genomes
LPS	Lipopolysaccharide
MHC	Major histocompatibility complex
mTOR	Mammalian target of rapamycin
NK	Natural killer

PAMP	Pathogen-associated molecule patterns
PBS	Phosphate buffered saline
PRR	Pattern recognition receptor
PTM	Post-translational modification
qRT-PCR	Quantitative real-time polymerase chain reaction
SCFA	Short chain fatty acids
SPI	Salmonella pathogenicity island
STRING	Search Tool for the Retrieval of Interacting Genes
TGF	Transforming growth factor
TLR	Toll-like receptors
TNF	Tumor necrosis factor
TTSS	Type III secretion system

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CHAPTER 1: INTRODUCTION

Foodborne illnesses cause health and economic burden in the United States annually affecting 48 million people every year. *Salmonella* is estimated to cause 1.2 million illnesses, 23,000 hospitalizations, and 450 mortalities annually in the US (1). One of the major causes of human gastroenteritis is *Salmonella enterica* (*S. enterica*) due to infected poultry products. Although improved control measures have been implemented, *S. enterica* still continues to present an issue every year in the US (2). One of the many reasons is due to the chickens' ability to co-exist with *Salmonella* without showing any outward clinical signs of distress, also referred to as disease tolerance (3). By looking at the gut health of chickens, there may be more to their ability to co-exist with *Salmonella* is currently known.

Chickens present a unique immunological perspective due to the early interaction between the gut immune system and the microbiome. Commercial broiler chicks are placed on used litter which raises the risk of the naïve gut microbiome of neonatal chicks to be colonized by pathogenic bacteria, like *Salmonella*, and take advantage of this susceptible environment (2,4–6). Recent findings have speculated a two-part infection occurring in the chicken ceca: early (4 to 48 h) and late (4 to 14 d) infections with *S.* Enteritidis with three distinct immune-metabolic (herein referred to as immunometabolic) phases post-infection: disease resistance, disease tolerance, and homeostasis (3,7). This two-part dysbiotic phenomenon may attribute to the disease resistance to infection tolerance stage, not to be confused with immune tolerance (3).

There is a lack of studies regarding the relationship of the metabolic and immunological changes occurring in the gut microbiota during *Salmonella* infection, especially in the chicken. While it is common knowledge that chickens infected with *Salmonella* do not manifest any obvious disease symptoms, the mechanism occurring in the host during infection has not been widely characterized. What is known are the basic steps of Salmonella pathogenesis in the infected host: attachment onto the mucous membrane, evasion of host defenses, multiplication to significant numbers at the site of infection, damage to the host, and transmission from the infected host to non-infected host (8). After oral ingestion by the host, *Salmonella* manages to survive in the acidic condition of the stomach due to an acid tolerance response (8,9). Once *Salmonella* makes its way to the intestine, the interaction with the mucosal surface is crucial for successful attachment. Inflammatory responses can occur in as little as few hours after oral ingestion. Not surprisingly, the damage to the host does not occur in the chicken. Instead, a state of persistence occurs, allowing for minimized host defense action. While this is not harmful for the chicken, it is not ideal downstream for human consumption (3).

While *Salmonella* in meat products are not desirable in the market, the producers have faced another challenge of curbing pathogens with antibiotics. In many countries, the use of antibiotic growth promoters (AGP) has been banned in animal feed, even at subtherapeutic levels. For over 60 years, bacitracin methylene disalicylate (BMD) and virginiamycin were widely incorporated at therapeutic levels in feed to prevent bacterial infections and to improve growth performance (10). Fluoroquinolones, such as nalidixic

acid and ciprofloxacin, have been used to control *Salmonella* but continual usage has resulted in the development of resistance from these pathogens (11). The Food and Drug Administration (FDA) has issued the Veterinary Feed Directive (VFD) over the recent years to ensure proper usage of antimicrobial additives in food production animals (12). As a result, subtherapeutic levels of AGP, such as BMD, must now be provided under veterinary authorization.

Antibiotics are observed to be anti-inflammatory in nature (13) but do not provide much effect on the cecal colonization of *Salmonella* in birds (14). There has been increased interest in utilizing plant-based compounds or phytobiotics as AGP alternatives, including tannins. Tannins can be found in many plant species, mostly in the inedible portions of the plant, such as the bark or wood. Immunomodulation refers to a dietary product or additive that possesses anti-infective properties so that the host immune response can be stimulated to enhance antimicrobial effects and to limit inflammation-induced tissue damage (15,16). The bioactive compounds found in tannins allow them to be an effective immunomodulatory additive that promotes health (17). Previous studies have been conducted to evaluate the functionality of different tannin species against pathogenic infections (18–20). These studies showed inhibitory bacterial activity by varying concentrations of tannins (between 0.5-1 kg/ton). Furthermore, tannins have also been shown to improve feed efficiency, growth performance, and intestinal health when 0.5-1 kg/ton of tannins are added directly into the poultry feed (21). When it comes to immunity, chestnut tannins exert pro-inflammatory immune responses but only temporarily when anti-inflammatory response

follows soon after (22). According to a review by Redondo et al (23), most of the *Salmonella* studies performed with phytobiotic effects were done *in vitro*, presenting a need for more *in vivo* studies.

Based on current information, this project will provide useful evidence about disease progression from an immunometabolic perspective to fill in the knowledge gaps. The current knowledge is that Salmonella persistence changes the immunometabolic profile of the cecum to establish itself and induce disease tolerance. There is also a growing need to find alternatives to antibiotics (ATA) in feed and chestnut tannins are promising candidates to fill in the current knowledge gap. Therefore, the objective of this work was to elucidate the mechanisms of how *S.* Enteritidis affects immunometabolic tissue phenotype change in early broiler growth. This objective was approached in a two-prong viewpoint by looking at different functional gut health modifiers currently utilized in the industry and their role in the affecting the host tissue phenotype: antibiotic growth promoters (AGP) and chestnut tannins (ChT).

CHAPTER 2: LITERATURE REVIEW

2.1. Poultry gut microbiota review

2.1.1. Poultry gut microbiota and its relation to host health

The gut microbiota is responsible for maintaining the integrity and function of the GI tract but also indirectly affecting other health processes. It is crucial for nutrient metabolism and the prevention of pathogen colonization. The gut microbiota has also the most complex and diverse bacterial community found in the body. In newly hatched chicks, the GI tract is considered naïve or unstable, which provides ample opportunity for pathogens to invade and establish colonization in the gut for persistent infection throughout the chicks' lifespan (5). As the birds mature, the resident microbial communities stabilize and increase in diversity and taxonomic richness (24). Bacteria are the dominant taxa in the animal gut microbiome, consisting of Bacteroidetes and Firmicutes (25). These two bacteria have been shown to affect energy balance, nutrient absorption, and metabolism dysfunction (26). A previous study noted the increase in richness and diversity between 3-6 weeks of age in the cecal microbiota of broilers, most likely due to the diversification of Firmicutes (24). Within the *Enterobacteriaceae* family, opportunistic bacteria like *Salmonella*, *Shigella*, and *Escherichia coli* are part of the resident gut microbiome community (5). Any dysbiosis event, usually from ingestion, can easily trigger changes from beneficial or benign bacteria to pathogenic bacteria. Dysbiosis can occur for a few days or become a slow accumulating event. The disruption of the normal microbiota would allow certain bacteria to take advantage of the

shifting energy source and rapidly colonize, outcompeting normal flora. The high prevalence of certain bacteria in the resident microbiota could alter the fitness to their advantage, potentially conditioning the chick GI tract to select for their colonization (5). Because of the heavy roles that gut microbiota plays in overall health, it is difficult to find a singular method that will encompass the role it plays throughout the body. Antibiotic intervention in humans modifies the gut microbiota so that once the antibiotic regime is finished, a new set of bacterial enzymes become active (27). As a result, the new set of enzymes promoted negative health consequences by favoring rapid assimilation of carbohydrates, inducing obesity or diabetes. Ultimately, a sub-optimal microbiota can induce a pro-inflammatory host response which would be detrimental to growth performance (28). With poultry production, this would greatly impact the feed conversion efficiency and growth performance due to altered gut microbiota (5).

2.1.2. Gut microbiota and their effects on immunity and metabolism

Beyond solely bacterial composition, another factor to consider is the host phenotype during gut modulation. In this case, phenotype is referring to the immune and metabolic alterations found on the tissue level. While studies have examined the host genotype as an important factor in affecting the gut microbiota composition, there is increased evidence that metabolism and immunity also play an influential role in gut microbiota in relation to gut health (5,29,30). This means the gut microbiota contributes to immunomodulation, affecting the innate and acquired immune system. Classic metabolic energy pathways, including AMPK and mTOR, are found to be linked to

immune cell function, specifically relating to CD8+ T cell functions (3). Therefore, this link may be key to determining the function of the gut microbiota and the role of immune cells in metabolism. While increased inflammation may affect the animal's performance level, appetite and muscle catabolism, the shifting gut microbiota of young broilers would adjust for the imbalance and lead to less inflammation and energy expenditure for the animal (13,31). The presence of these signaling molecules is important for the epithelial cells at the site of infection and stimulation of macrophages to produce nitric oxide radicals (32). Recent work has demonstrated that certain antagonists allow pathogenic *Enterobacteriaceae* members including *Salmonella* spp. to upregulate the expression of antimicrobial proteins to survive (33). Thus, this disruption of the microbiota increases the pathogen's drive to survive by changing the state of inflammatory response. Furthermore, the presence of *Enterobacteriaceae* has been shown to influence the metabolites expressed in the gastrointestinal tract (GIT), especially with short-chain fatty acids (SCFA) (30). Many SCFA are important for energy production for epithelial cells and immune cells including T cells and macrophages (34). Previous works have demonstrated the beneficial effects of SCFA in the gut, especially butyrate which serves as an energy source for epithelial cells, stimulates mucus production, and modulates the immune system while promoting pathogen control (35). Therefore, manipulating the nutritional composition of feed has the potential to improve host development and improve the immune system (36). Overall, we are aware of the GIT acting as the interface between diet, host, and gut microbiota while also serving essential roles in the animal's immunity and health.

However, more studies are needed to focus on the avian model to fully understand how it will impact the meat industry.

2.1.3. Functional gut health modifiers

Functional gut health modifiers are defined as therapeutic interventions aimed at altering the host immune response to effectively thwart disease states (27). The involvement of the gut microbiota to mount host defenses to modulate the immune system is already in use for human medicine (27). One approach that is currently being explored by our group involves host-directed immunomodulatory therapies to improve host health. Unfortunately, previous studies have only concluded on the direct effect of an additive on immunity, rather than considering how it affects the gut microbiota and its downstream effects (36). In the poultry industry, functional gut modifiers are also in use, such as BMD and phytobiotics, to improve poultry health and growth development. The usage of a functional gut health modifier, such as BMD, has been noted to improve performance by reducing inflammatory markers (13). These dietary modulators are used to alter the gut microbiota to protect from pathogens and modulate the immune system as well (37). Most AGP target Gram-positive bacteria since they are usually associated with poorer animal performance and growth (24). Furthermore, they may promote growth, proliferation, or intactness of beneficial and resident bacterial gut communities to confer protection against pathogens, such as *Salmonella enterica* (38). What is important to note is that to promote an optimal immune system, the host needs a balanced pro-inflammatory and anti-inflammatory response. The suppression of pro-

inflammatory responses does not always reap benefits to the host immunity. In fact, it may make the initial immune response less effective while allowing pathogens to proliferate on the opportunity of imbalance (28). However, most of these studies have been performed in vitro, which concluded in variable results of AGP and its effects on phagocyte or immune cell functions (28).

Currently in the US, antibiotic usage in poultry feed is facing scrutiny due to antibiotic resistance and increased consumer awareness (21). The decline of AGP usage requires an alternative functional gut health modifier to be utilized by producers. As a result, phytobiotics have become ideal ATA due to the availability and accessibility. Phytobiotics have also been shown to enhance animal performance and nutrient availability, similar to AGP (17). The usage of phytobiotics has been included for many years as a natural growth promoter in livestock industries, such as the inclusion of herbs, spices, essential oils, and oleoresins (10,39). Although studies have shown enhanced immunity and health benefits of some phytobiotics, the mechanism of how they function is not clearly understood (21). Current speculation of the antimicrobial action of phytobiotics suggests disruption and/or modification of the cell membrane of the pathogen (39). Another study determined that phytobiotics may stimulate the immune system by activating lymphocytes, macrophages, and natural killer (NK) cells (40). A future ATA should consider the following effects: (1) optimal dose for immune effect; (2) the variations in active compounds in plant-derived products; (3) safety of these compounds for animal and human consumption; and (4) the long-term effects on antimicrobial resistance (21). This promotion of host health via modulating the gut

microbiota is a widely unexplored field in poultry and requires more studies to be conducted.

2.2. *Salmonella enterica* Enteritidis biology in the chicken

2.2.1. *Salmonella* and its influence on food production in the US

Salmonella enterica continues to be the leading cause of disease in food production animals and public health globally (9). Outbreaks commonly occur in meat, vegetable, and egg products worldwide. This leads to a global economic burden resulting in $3.5 billion in loss of production and healthcare costs (41). There are over 2,500 known serotypes of *S. enterica*, two of which are widely studied for their prevalence in humans and food production animals: serotypes Typhimurium and Enteritidis (*S.* Enteritidis) (42). Due to the diversity of *Salmonella* spp., the control of infections has been difficult, in addition to reporting trends varying by states (43). Since the early 1960s, there has been an ongoing incidence of antimicrobial resistance in *Salmonella* strains particularly affecting the meat production industry (44). Despite improved safety methods, testing, and hygiene practices, infections continue to rise annually in industrialized and developing nations (44). Although improved antimicrobials were introduced, such as fluoroquinolones and nalidixic acid, there is growing resistance reported in *Salmonella* isolates globally. Currently, *Salmonella* spp. are resistant to most traditionally used antimicrobial agents such as ampicillin, chloramphenicol, and trimethoprimsulfamethoxazole (44). Therefore, *Salmonella* continues to pose a challenge to the poultry production and public health realm.

2.2.2. Salmonella pathogenesis in chickens

When humans or mice are infected with *S. enterica* from the ingested route, the bacteria manage to survive gastric stomach passage and onto the small and large intestine's mucosal layer. First, *Salmonella* attaches to the mucosal membrane of the small intestine. Through an evolved series of mechanism, *Salmonella* can evade host defenses and multiply to a significant number at the site of infection or spread to other organs, using M cells on Peyer's patches to enter or via dendritic cells. They are then able to penetrate the intestinal epithelial cells depending on the pathogenicity islands (SPI) utilized for progression. This evolutionary mechanism by *Salmonella* is incredible because it is essentially inducing phagocytosis to gain access into the epithelial cell of the host. Both SPI-1 and SPI-2 encode type III secretion system (TTSS) which acts as a syringe full of virulence factors to successfully establish colonization (45). Once they are involved in the Peyer's patch, *Salmonella* will induce apoptosis of the phagocyte it is encapsulated in (whether it be dendritic cells or macrophages) to escape. This causes caspase-1 to cleave pro-inflammatory cytokines IL-1B and IL-18 and to be released during apoptosis (46). This stimulates NK cells, macrophages, and T cells to produce IFN-γ in order to activate bactericidal activity and Th1 development (46). The build-up of inflammatory response may be responsible for damage to the intestinal wall and may contribute to the clinical manifestations in mammals (47).

Similar to mammals, chickens are infected via the fecal-oral route and once the pathogen reaches the distal ileum and/or cecum, they will attempt to outcompete the host

microbiota to establish infection (9). It manages to enter the lamina propria via Peyer's patches which will initiate the cascade of pro-inflammatory cytokines released (46). The ceca of the chickens are popularly observed sites of *Salmonella* colonization. The pathogenesis in chickens is similar to humans until the point where *Salmonella* infiltrates the epithelial cells. Similar to mammals, chickens also utilize SPI-1 and SPI-2 to evade host defenses although SPI-2 seems to be playing a role in the colonization of the spleen while SPI-1 plays a role in both cecum and spleen (48). Furthermore, a study looking into TTSS mutation in Typhimurium indicated that infection still occurs systemically in the chicken (49). Once the macrophages have ingested *Salmonella*, there is an upregulation of pro-inflammatory cytokines IL-6 and IL-1 to the site of infection (50). The presence of these pro-inflammatory cytokines leads to infiltration of heterophils into the GIT.

Salmonella Enteritidis differs from the other *Salmonella* strains because of its ability to tolerate acidic and oxidative environments to persist effectively in the chicken GI tract, thus creating a persisting food safety issue (51). According to Kogut and Arsenault (3), chickens do not seem to display the classic strong pro-inflammatory response as mammalian species. Colonization of *S.* Enteritidis generates an influx of heterophils and macrophages as the pro-inflammatory cytokines and chemokines continue to be released (42). However, heterophils alone do not provide enough protective response against *Salmonella*, causing a significant decrease in pro-inflammatory cytokines which allows *Salmonella* (particularly seen in Enteritidis) to persist in this environment. This results in a metabolic reprogramming of the phenotype

of the tissue (3). After this point, there is much speculation as to what occurs in the birds, and the only inferences that can be made are based on murine studies.

2.2.3. *Salmonella* persistence (disease resistance to disease tolerance)

Under physiological homeostasis, broiler chicks contain similar gut microbiota bacterial composition as humans: mostly consisting of obligate anaerobic Gram-negative bacteria and facultative anaerobic bacteria (2). At this point, the gut bacteria and the resident microbiota coexist but when pathogens invade, the resident microbiota can generate the proper immune response without causing overt damage to the tissues. However, under dysbiotic conditions, there is a shift in the gut microbiota community and an increase in the population of facultative anaerobes, causing a competition for resources and a disruption in the immunity. The act of the host defense responses of sensing and eliminating pathogens is often referred to as “disease resistance” (52). It is theorized that this mechanism alone does not establish a negative impact on pathogenic fitness but works in synchrony with the resident microbiota to respond to pathogens. As a result, disease tolerance, the ability to endure microbes and minimize physiological damage to the host, becomes established within the host (52,53). While tolerance seems ideal, it is also the act of remaining neutral to positive impact on microbial fitness, which may explain the co-evolution of host-microbe interactions (52). Disease resistance and tolerance have been well documented in murine models: the longer the pathogen persists in the host, tolerance defenses will prevail to balance the host-pathogen mutualism (54). The release of anti-inflammatory cytokines during the tolerance phase promotes host-

pathogen interactions to persist by minimizing the damage by the immune system in response to the pathogen (54).

Parallel with the immune response, there is a metabolic component that plays a part with the microbiota during disease tolerance. Microbes need metabolic byproducts to support their survival and ensure success in colonization; this is done by changing the environment and nutrient supply during the infection process (52). The gut microbial communities that are in close proximity to host tissue work in a close relationship, therefore, when dysbiosis occurs, the gut epithelial cells of the tissue becomes vulnerable to pathogenic or opportunistic attacks (54). The host immune response will contribute to tissue damage while elevating an inflammatory response to attempt to remove the pathogen. Eventually, the host will return to an energy homeostasis, which only aids the host tolerance of the microbiota (55). However, the immune responses to rid of the pathogen cost the host significant energy which will affect the gut microbiota and metabolic products downstream (54). A study performed with inflammasome-deficient mice suggested that inflammasome activation by the microbiota affects the energy consumption which contributes to the onset of tolerance (56).

Similar to mice, *Salmonella* takes advantage of this shifting opportunity to successfully establish persistence in the GI tract by evading chicken immune responses. As the pathogen continues to proliferate, there is an increase in signaling for pro-inflammatory cytokines such as IL-1B and IL-6 (2). Concurrently, the metabolism is altered due to the immunological response. Our group has performed numerous studies to demonstrate the similar resistance to tolerance switch in the chicken model, proposing

that *Salmonella* infection can be separated into 3 distinct stages of host defense strategies as characterized by immunometabolic responses: 1) stage 1, disease resistance; 2) stage 2, disease tolerance; 3) stage 3, homeostasis. The disease resistance stage is mediated by mTOR protein synthesis, due to the pro-inflammatory state (3).

Stage 1 or disease resistance is when *Salmonella* invasion of the GI tract induces inflammation due to an elevated response of pro-inflammatory cytokines (57). This inflammatory response stays elevated until days 3-4 post-infection when there is a reduction of pro-inflammatory response. Concurrently, the tissue metabolism is affected due to the influx of heterophils and other immune cells that cause an increase in fatty acid synthesis, glycolysis, and protein synthesis (58). The presence of these polymorphonuclear leukocytes rapidly depletes the environmental oxygen supply, promoting the influx of hypoxia-inducible factor-α (HIF1α) to resolve the inflammation state (59). Activation of HIFIα inhibits mTOR activity which reduces inflammatory responses which would regulate further tissue damage. This initial inflammatory response, designated by Kogut and Arsenault (3) as disease resistance in chickens, aids in the development of a protective acquired immune response to clear GI infections.

Stage 2 or disease tolerance is classified by the presence of increasing regulatory cytokines such as IL-10 and TGF-β at the beginning, developing an anti-inflammatory environment (3). The expansion of T_{reg} cells in the ceca also increases IL-10 mRNA expression to ultimately help with the clearance of *Salmonella* in the host (60). Our group has further confirmed the definitive resistance to tolerance phenotype switch by discovering the two altered immune signaling pathways: JAK-STAT and T cell receptor

pathways (58). Concurrently, the alterations to the immune response affect the metabolism in the ceca of *Salmonella*-infected chickens, undergoing an initially mTOR-mediated pathway to AMPK-directed oxidative phosphorylation (61). It appears *Salmonella* prefers macrophages that utilize oxidative metabolism for energy which signals the activation of NLRP3 inflammasomes and pyroptosis (3).

The 3rd stage (homeostasis) occurs after day 4 post-infection with similar parameters as the tolerance stage. This was confirmed by the persisting presence of IL-10 and TGF-β mRNA levels and no changes observed in the metabolic or immune signaling pathways in *Salmonella*-infected ceca (58). Conditions that will affect the energy homeostasis status, such as anorexia and malnutrition, reduces metabolic substrate availability to maintain the energy demands of growth development (52). More studies need to be conducted to determine more canonical parameters for this stage.

2.2.4. Current *Salmonella* knowledge and its influence on poultry immunity

While there are increasing information available on murine immunity against *Salmonella* infection, there are still limited information on the immune response on livestock animals, including chickens. Chickens, as our main study model, possess pathogen recognition receptors (PRR) compared to mammals, especially when looking into Toll-like receptors (TLR) (36). Different TLRs will initiate different innate immune responses in animal immunity. For example, certain TLRs will recognize pathogen associated molecule patterns (PAMP), signaling the innate immunity to respond accordingly such as heterophil activation (62). Chicken TLR2 type 2 has been found to

respond to LPS and induce oxidative burst, serving a potential role in the chicken's innate immune response. PAMP are only found on pathogens, thus it is a feature that the innate immunity should recognize as non-self. PAMP are recognized via cell-extrinsic pathway involving TLRs and cell-intrinsic pathway involving inflammasome creation (36). Within the cell-extrinsic pathway, the bacteria are detected by dendritic cells through TLRs where the PAMP are formed into phagosome, which is then presented on the MHC II complex and induces cytokine and co-stimulatory molecules for T lymphocyte activation. The cell-intrinsic pathway also occurs on dendritic cells and activates T lymphocytes but it can present on both MHC I and II depending on the type of PAMP detected. These are crucial for host defense against viruses, bacteria, parasites and fungi but need to be strictly controlled because excessive inflammation will be harmful to the host (may cause pyroptosis) (62). The inflammasome is also important for the activation of an enzyme (caspase) that initiates the stimulation of pro-inflammatory cytokines. Once PAMP are recognized, different signals are induced: 1) mediation of inflammatory responses by IL-1, TNF-α, IL-6 and IFNs; 2) mediation of costimulators of T-cell stimulation; and 3) mediators of effector cell functions such as IL-4, IL-10 and TGF-β and IFN-γ (36,62,63).

The mechanism of intestinal colonization is not the same as intestinal disease persistence. As mentioned before, once colonization occurs in the "early phase of infection" (usually upon day of infection to day 2 in broilers), the host defense switches from disease resistance to a disease tolerance phase (usually upon day 3 or 4 in broilers) (15,29). This type of tolerance occurs when *Salmonella* persistence cannot be rid of;

therefore, the host defense limits the damage done to tissues (by itself and the pathogen) instead of continuing an effort to keep increased pro-inflammation which would further create tissue damage. After day 4 post-infection, the pro-inflammatory immune response is largely reduced to the presence of anti-inflammatory cytokine release and the host defense no longer seems to recognize *Salmonella* as a pathogenic threat, letting it persistently colonize in the ceca. In terms of metabolism, the mTOR protein synthesis pathway is turned off in the ceca and switches to an AMPK pathway, which is indicative of an anti-inflammatory state. This metabolic and immunological phenotypic change creates an ideal environment for *Salmonella* to persist.

From day 1 to day 16 of age in chicks, IL-17 and IL-22 are induced by the presence of *S. enterica* Enteritidis infection with the receptor of the respective cytokines present across the days of infection (2). Induction of IL-17 in the first week of the chicken's life stage is thought to result in an influx and differentiation of Th17 cells in response to microbiota colonization (64). If the birds reencounter *S.* Enteritidis by day 16, there is an increased Th17-dependent resistance that protects the birds from *Salmonella* damage in the liver and spleen and a reduced cytokine expression of IL-22 and IL-17 in older birds (2).

2.2.5. *Salmonella* and its effects on the gut microbiota

As mentioned before, *Salmonella* can colonize post-hatch chicks and affect their gut microbiota to where beneficial microbes in the intestine and ceca cannot establish properly (65). They can contract *Salmonella* from external factors such as water, food,

and the litter. Many of the taxa present in the chicken GIT already include *Salmonella* spp. as part of the gut resident microbial community (26). The ability of *Salmonella* to cause disease depends on the age of the bird, immune status or bacterial load of the strain (9). Generally, older birds are resistant to infection or colonization of *Salmonella.* In young birds, the infection with *Salmonella* post-hatch can be detrimental to the overall development of the gut microbiota, resulting in an inflammatory response of the GI tract which influences the microbiota composition (66,67). Videnska et al. (66) also concludes that the changes in the cecal microbiota after *Salmonella* challenge can be characterized as an indirect result of the infection, and not as a selected evolutionary mechanism. A fully functional, mature microbial community is vital for the host's ability to confer protection against pathogenic colonization to then ensure optimal growth and development (67).

2.3. Kinome and post-translational modification review

2.3.1. Importance of kinomics

Studying phosphorylation events provides the mechanism of post-translational modification (PTM), which offers insight in cellular and tissue phenotypic mechanisms (68,69). These are catalyzed by kinases which eventually provide phosphorylation events. Peptide arrays for kinomic analysis have already been widely utilized across scientific disciplines (69–72). Peptide arrays for kinomic analysis utilize peptides which represent kinase target sites which are then synthesized, and its activity is measured by the array (71). They are already heavily utilized in detecting kinase activity associated

with diabetes, inflammation, cancers, and other disorders (73). The kinome array provides functional phenotype data, indicating the changes within tissue metabolism and immune response to an infection (29). Recent advancements have provided a species-specific peptide array for livestock species, including poultry (69,74). Protein phosphorylation determines its functional importance by regulating critical parameters of protein activity, stability, and interactions (73). The peptide array can provide immunometabolic phenotype data as well as functionality data. The peptides selected for the array fall into one of three categories: 1) peptides that could be considered part of the central cellular signaling hubs (for example, AKT, MAPK, PI3K), 2) peptides involved in the innate and adaptive immune systems; and 3) peptides involved in metabolic processes (for example, glycolysis, fatty acid synthesis, protein catabolism, protein synthesis) (75).

Although the field of transcriptomics is highly popular, the drawback of transcriptomics is that there are several processes and potential disruptions that can occur before the final active protein is generated. These include gene silencing, mRNA stability, translation, translational efficiencies, protein turnover, sequestration of enzymes from substrates, and the multitude of post-translation modifications (72). Although the study of the genome and genes is important, genes provide data on only what is present, rather than dynamic data across complex disciplines, such as host-pathogen interactions, immune responses, metabolic response, and affected effector cells (3). In other words, the genome is constant, but the proteome varies and is dynamic even within tissues. Gene transcription does not necessarily result in translation due to

mutations or silencing events before the production of a protein (75). A single protein can activate enzymatic activity on one site while deactivating or phosphorylating another site (75). For example, c-Src requires a combination of phosphorylation and dephosphorylation to be activated simultaneously. Therefore, it is important to be able to track phosphorylation events and its activity downstream.

2.3.2. Chicken-specific kinome arrays

Our group has contributed to the design of chicken-specific kinomic peptide array through the curation of phosphorylation databases such as PhosphoSitePlus (www.phosphosite.org) and PhosphoELM (phospho.elm.eu.org). While the human and mouse phosphorylation databases are well-documented, these databases need to be utilized for the design of chicken-specific arrays (75). Out of the 200,000 phosphorylation target sites, about 70-75% were exact matches to the chicken proteomes (76). However, this has proven to be an advantage because there would be no potential cross-reactivity between human phosphorylation target sites and chicken kinases. Therefore, the species-specific kinome array also has an advantage over antibody arrays for immune validation purposes. Some of the classic energy metabolic sensors, such as AMPK and mTOR, are linked to innate and adaptive immunity (77). By elucidating the kinase activity, insights into identifying specific biomarkers will provide future therapeutic targets (69). Previously, our group has determined that *Salmonella* affects the fat deposition and carbohydrate metabolism in challenged chickens with the utilization of the kinome array (29). Thus, this integrated array demonstrates the importance of

combined immunity and metabolic data on the animal's overall health and growth performance.

2.4. Phytobiotics and immunity

2.4.1. Phytobiotics introduction

Feed additives have been known to indirectly modulate the intestinal microbiota by altering host immunity (78). The utilization of polyphenolic compounds (or phytochemicals) as pharmaceutical alternatives has been extensively studied over the years, including flavonoids, phenolic acids, and tannins, to improve host immunity and development (79). Phytochemicals are plant-derived bioactive compounds that enhance health and have been regarded in traditional medicine for its antimicrobial and antiviral activities in humans (21). By moving away from antibiotics in livestock production, there is a growing interest from the poultry industry to find plant-based alternatives to replace antibiotics in feed. There has been increased interest in utilizing plant-based compounds or phytobiotics as ATA (37,80). These phytobiotic feed additives are commonly used in traditional treatments although the mechanisms of how they function are widely unknown (81). The current knowledge, as summarized by Lillehoj et al. (21), is that the active ingredients in phytobiotics alters the host microbiota, providing antimicrobial activities against pathogens, and reduces oxidative stress to improve overall health of the animal. A review published by Diaz Sanchez et al. (39) tabulates phytobiotic studies with effects on performance and inclusion rate. Extensive studies have been performed in other food production species, particularly in swine, which exhibited evidence of phytobiotics as

likely alternatives to antibiotics to improve growth performance and health (20,82,83). Ruminant production has increased interest in growth promoters because of the efficient utilization of energy during rumen fermentation (84). Although the data vary between each type of phytobiotic classification, the ideal antibiotic alternative would alter the host microbiota to guide protein and lipid metabolism, promote effective nutrient utilization, and prevent harmful infections to the host (85). Furthermore, the site of *Salmonella* and other foodborne pathogens is the cecum, located at the posterior end of the GIT. This poses another challenge for the development of an ATA because the phytobiotic must retain its activity during the movement through the GIT (39). One of these promising phytobiotics extensively studied in the literature is tannins, a readily found plant-based compound with antimicrobial activities and growth performance promotion (21).

2.4.2. Tannins

Tannins can be found in many plant species, mostly in the inedible portions of the plant such as the bark or wood (17,86). The previous knowledge was that tannins possess anti-nutritional effects in livestock species but with new evidence, the benefits show promising results across livestock species depending on the dosages and quality of tannins in the feed (37,87). Within the tannin subcategory, there are two major classes: hydrolysable tannins and condensed tannins. It is believed that tannins may exert their biological effects in two different ways: (1) as an unabsorbable (condensed) structure with binding properties which may produce local effects in the gastrointestinal tract (antioxidant, radical scavenging, antimicrobial, antiviral, antimutagenic and antinutrient

effects), or (2) as absorbable (hydrolysable) tannins (probably due to low molecular weight) and absorbable metabolites from colonic fermentation of tannins that may produce systemic effects in various organs (88). Studies have shown that tannins are not only effective immunomodulatory additives (17) but also enhances metabolic functions, such as decreased lipid oxidation (89) and increased beneficial fatty acids (90).

The active ingredients in phytobiotics alter the host microbiota, providing antimicrobial activities against pathogens, and reduce oxidative stress to improve the overall health of the animal (21). Previous studies have revealed that incorporating low concentrations of tannins in diets improved the health status, nutrition, and performance of the animal (21,86,91). For many livestock species, high concentration of tannins in the diet reveals the opposite: decreased overall immune function, reduced weight, and reduced nutrient digestibility (91,92). Chestnut tannins (*Castanea sativa* Mill) and quebracho tannins (*Schinopsis lorentzii* Engl.) have had the same promising results as noted previously, with the addition of antibacterial, antioxidant, and antiviral potential (86,93). It has been observed that both types of tannins have been successful in controlling or diminishing the incidence of foodborne pathogens, such as *Salmonella* (23), *Campylobacter* spp. (94), and *Clostridium perfringens* (23,95).

Previous studies have shown the promising antibacterial effects of both hydrolysable and condensed tannins, although their antibacterial activities may differ based on molecular weights and concentrations provided in feed (37,94,95). Tannic acid is a hydrolysable polyphenol found in tannins, with evidence of beneficial effects on health due to its antioxidant properties, such as disruption of virus mechanism as shown

in human immunodeficiency virus (HIV), *Herpes simplex* virus (HSV), and Norovirus models (96,97). In terms of how tannins affect the immune system, the mechanism is still widely unknown. Some heavily researched tannins such as Oenothein B, an isolated ellagitannin from *Epilobium angustifolium*, has been studied for its ability to inhibit or promote inflammatory responses by phagocytes (98,99). This compound also activates human CD3+ T cells, γδ T cells, CD8+ T cells, and CD3-/CD56+ NK cells, which led to the production of IFN-γ (99). In another study by Ramstead et al. (100), the immune response differs based on the age of the human, primarily that T cells of younger humans produce significantly less IFN-γ and granulocyte macrophage colony-stimulating factor (GM-CSF) than adults in response to ellagitannins. Similar results have been shown in murine cells, with the increased frequencies of CD8+ IFN-γ T cells (101). These results show the immunomodulatory potential of tannins and their ability to contribute to antiviral activity. However, it is important to consider variables, such as the age of host or ingested concentration, when interpreting the response of tannins on the immune system.

There are currently a growing number of publications looking into the efficacy of tannins with antimicrobial potential by improving the host immunity. It has been established that tannins exhibit anti-inflammatory effects in the host due to lowered counts of lymphocytes (102–104). This protective activity reduces inflammation and tissue damage caused by infectious agents (103). While this is not specific to chestnut or quebracho tannins, several studies have shown that treatment with ellagitannin geraniin stimulates murine macrophages to secrete pro-inflammatory cytokines including tumor

necrosis factor (TNF) and interferon (105). Therefore, the potential for immune modulation by chestnut or quebracho extracts are highly probable in animal models. Another recent *in vivo* study performed by Ramah et al. (91) demonstrated that lower concentrations of tannins in basal diets improved immune response, increasing CD4+ CD8+ cells and γδ T cells in the spleen of chickens compared to the control group. Furthermore, chickens fed lower concentrations of tannins had increased IFN-γ mRNA expression level stimulating the immune responses reflected by the increasing γδ T cell population (91). There are currently mixed reviews on the immunostimulant or immunosuppressant effects of tannins, requiring further clarification in studies of how tannins modulate the immune response (79).

Therefore, there is a need for more host-phytobiotic interaction studies to understand the mechanism of tannins' effect on immunity. The research on this topic has gained recent popularity due to finding effective alternatives to antibiotics in production animal feed. Further studies should be conducted to elucidate the full mechanism of how tannins may act on microbial infections.

CHAPTER 3: "A ROLE FOR THE MICROBIOTA IN THE IMMUNE PHENOTYPE ALTERATION ASSOCIATED WITH THE INDUCTION OF DISEASE TOLERANCE AND PERSISTENT ASYMPTOMATIC INFECTION OF SALMONELLA IN THE CHICKEN."

3.1. Introduction

Foodborne illnesses cause health and economic burden in the United States annually affecting 48 million people every year. One of the major causes of human gastroenteritis is *Salmonella enterica* Enteritidis due to infected poultry products, accounting for 40-60% of all reported cases (43). Although improved control measures have been implemented, *S. enterica* still continues to present an issue every year in US livestock (2). One of the many reasons is due to the chickens' ability to co-exist with *Salmonella* without showing any outward clinical signs of distress, also referred to as disease tolerance, and increased antimicrobial resistance due to overuse of antibiotic growth promoters (AGPs) (3). Although the mechanism of how AGPs improve animal performance is still unclear, Diaz Carrasco et al. (37) speculate it is through intestinal microbiota modulation, specifically in the ceca for the chicken, whether it is dietary-related or pathogen-related. There needs to be studies focusing on identifying the mechanism of how broad-spectrum antibiotics, such as bacitracin methylene disalicylate (BMD), work in the host gut towards improving the overall health of food production animals. Therefore, focusing on the gut microbiota may provide insights on improved mechanisms and health management strategies. Increasing studies are showing the importance of the gut microbiota and its role in

digestion, host immunity, immune protection, and protection against pathogen colonization (106). Recent studies have reported the ceca containing the most diverse bacterial populations in the GIT: phyla *Bacteroides, Proteobacteria* and Firmicutes are the most dominantly observed (26,37,106). Many of the taxa present in the chicken GIT are of human relevance, including *Salmonella* spp. and certain *Campylobacter* spp. (26).

Chickens present a unique immunological perspective due to the early interaction between the gut immune system and the microbiome. Commercial broiler chicks are placed on used litter which raises the risk of the naïve gut microbiome of neonatal chicks to be colonized by pathogenic bacteria, like *Salmonella* Enteritidis (*S.* Enteritidis), and take advantage of this susceptible environment (2,4–6). Recent findings have speculated a phenomenon occurring in the chicken ceca with early (4 to 48 h) and late (4 to 14 d) infections with *S.* Enteritidis, resulting in three distinct immune-metabolic phases: disease resistance, disease tolerance, and homeostasis (7,36). There is a lack of studies regarding the symbiosis between the metabolic and immunological (immunometabolic) changes occurring in the gut microbiota during *Salmonella* infection, especially in the chicken. Once *Salmonella* enters the intestine, the interaction with the mucosal surface is crucial for successful attachment. Inflammatory responses can occur in as little as few hours after oral ingestion. Not surprisingly, the damage to the host step does not occur in the chicken. Instead, a state of persistence occurs, allowing for minimized host defense action. While this is not harmful for the health of the chicken, it is not ideal downstream for human consumption. This phenotypic alteration event has been theorized as a survival mechanism of *Salmonella* in poultry to minimize host defenses (3).

Therefore, the objective of this study was to evaluate the role of the microbiota in the immune phenotype changes induced by *S.* Enteritidis infection using subtherapeutic levels of BMD. The results from this study will provide crucial perspectives on how broad range antibiotics act on the ceca, especially during a *Salmonella* infection, and improvements on poultry intestinal health.

3.2. Materials and Methods

3.2.1. Experimental animals, housing, and treatments

All experiments conducted were in accordance with the guidelines set by the United States Department of Agriculture Animal Care and Use Committee (IACUC #2019-003). By-product broiler eggs (N=112) were obtained from a commercial hatchery. The fertile eggs were incubated (GQF Manufacturing Company, Savannah, GA; Jamesway Incubator Company, Inc., Ontario, Canada; or Petersime Incubator Co., Gettysburg, PA) and maintained at wet and dry bulb temperatures of 32°C and 37°C, presented as relative humidity. After 10 days of incubation, the eggs were candled; non-fertile and non-viable eggs were discarded. The viable eggs were returned to the incubator until day 18 when they were transferred to hatchers (Humidaire Incubator Company, New Madison, OH) and maintained under the same temperature and humidity conditions until hatch. At hatch, the chicks were randomly distributed into one of four groups (28 chicks/group): T1) non-infected birds fed a broiler starter diet alone; T2) *S.* Enteritidis challenged birds fed a broiler starter diet alone; T3) non-infected birds fed a broiler starter diet containing bacitracin at the inclusion rate of 50 g/imperial ton; T4) *S.*

Enteritidis challenged birds fed a broiler starter diet containing BMD at the inclusion rate of 50 g/imperial ton (Zoetis Inc., Parsippany, NJ, USA).

All chicks were randomly distributed into each group in pens with fresh pine shavings, water, and starter diet *ad libitum.* They were maintained in BSL-2 isolation units under 96 h light and then followed by 18 h light and 6 h dark until the end of the study. The temperature in the room was held between 90ºF to 95ºF (temperature decreasing to 90ºF by day 10). Birds were monitored daily for the entire experimental period. This study was repeated three times under identical parameters.

3.2.2. Bacteria preparation

Upon hatching, all chicks were orally challenged with 10^{6-8} CFU/0.5 mL *S.* Enteritidis or mock challenge with 0.5 mL sterile 1xPBS. This *Salmonella enterica* serovar Enteritidis isolate was obtained from the National Veterinary Services Laboratory (Ames, IA, USA), selected for resistance to novobiocin (25 ug/mL) and nalidixic acid (20 ug/mL) in tryptic soy broth (Difco Laboratories, Sparks, MD, USA). The viable cell concentration of the challenge dose for each experiment was determined by colony counts on XLT4 agar base plates with XLT4 supplement (Difco Laboratories, Sparks, MD, USA) and nalidixic acid and novobiocin (XLT-NN).

3.2.3. Bacterial enumeration and detection

For bacterial enumeration, the cecal contents (0.25 g/bird) were collected, serially diluted to 1:100, 1:1000, 1:10000, 1:100000, and plated onto XLT4 for

Salmonella detection. These plates will be incubated at 37ºC for 24 hours for colony counts. For the enrichment, 100 ul of the cecal contents were pre-enriched in Rappaport-Vassiliadis broth for 24 hours at 37ºC.

3.2.4. Sample collection and processing

On each necropsy day (day 2 and 4, post-infection), 14 birds/group were randomly selected and euthanized via cervical dislocation. The cecal contents were collected for bacterial enumeration and microbiome analysis. Half of the ceca were collected and snap frozen in liquid nitrogen for kinome and the other halves were stored in RNALater for gene expression studies. This trial was repeated two more times for three total separate experiments.

3.2.5. Microbiome sequencing

For the microbiota studies, the remaining contents from each ceca (ranging from 300-500 mg per cecal sample) were submitted to a core sequencing facility at the University of Arkansas, Fayetteville (N=14/treatment/day/experiment; 336 cecal contents submitted total). DNA was extracted from the ceca samples taken at the indicated time points (day 2 and 4) using the Qiagen Qiamp Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany). The DNA purity was assessed and then the DNA samples were diluted to 10 ng/mL. The paired-end sequencing libraries were prepared by targeting the hypervariable region 4 of the 16S ribosomal RNA with PCR primers containing the linker and adapter sequence. The libraries were assessed for qualitative

and quantitative homogeneity, and then sequenced using the Illumina MiSeq platform as previously described (107).

3.2.6. Microbiome bioinformatic analysis

Data sequences were uploaded onto BaseSpace (www.basespace.illumina.com) (Illumina, San Diego, CA, United States) to determine sequence run quality and run completion. De-multiplexed data were downloaded locally and uploaded onto QIIME2-2018.8 via the Casava1.8 paired-end pipeline. All data analysis on QIIME2 were conducted using the q2cli interface. Data were visualized and then trimmed in DADA2 using the chimera consensus pipeline. Alpha and beta diversity were then computed via the QIIME phylogeny align-to-tree-mafft-fasttree methodology, and analyzed for all available metrics of alpha and beta diversity via QIIME diversity core-metrics-phylogenetic, with a sampling depth of 14,000 reads for both diversity and alpha-rarefaction analysis. Taxonomic assignment was conducted using the QIIME feature-classifier classify-sklearn Bayesian methodology with the QIIME2-2018.8 SILVA database. PERMANOVA was used to calculate statistically significant differences in alpha and beta diversity. Nonparametric test ANOSIM was used to compare the similarity between the bacterial composition within treatments using UniFrac.

3.2.7. Real-time quantitative RT-PCR

The immune genotyping portion was quantitated by gene expression studies, through a TaqMan based assay adapted from Eldaghayes et al. (108). Total RNA was

extracted using a Qiagen RNeasy® Plus kit (Germantown, MD, USA) and evaluated with a NanoDrop™ 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Ceca stored in RNALater were used for RNA isolation with the Qiagen Rneasy Plus Kit. The ceca were cut longitudinally to expose the lumen and any remaining fecal matter was gently removed with forceps as to not disturb the mucosal layer.

Cytokine mRNA expression levels were ascertained using RT-PCR with 28S as the reference gene. The RNAs were stored in -80°C until plate setup. The cytokines IL-1B, IL-6, IL-10, IFN-γ, and TNF-α were quantified utilizing the Eldaghayes et al. method (108). Primer and probe sequences (**Table 1**) for amplification have been described previously by Kogut et al. (109) and Kaiser et al. (50). The plates were run in the Applied Biosystems ABI StepOne Plus PCR system (ThermoFisher Scientific, Waltham, MA, USA) with the previously stated TaqMan Assay under the following conditions: one cycle of 48°C for 30 min, 95°C for 20 s, and 40 cycles of 95°C for 3 s and 60°C for 30 s. Normalization was carried out against 28S rRNA, which was used as a housekeeping gene. To correct for differences in RNA levels between samples within the experiment, the correction factor for each sample was calculated by dividing the mean threshold cycle (Ct) value for 28S rRNA-specific product for each sample by the overall mean Ct value for the 28S rRNA-specific product from all samples. The corrected cytokine mean was calculated as follow: (average of each replicate × cytokine slope)/(28S slope × 28S correction factor). Fold changes in mRNA levels were calculated from mean 40 Ct values. Each sample was run in triplicates for technical replication.

RNA target no.		Probe/primer sequences	Accession
28S	Probe	5′-(FAM)-AGGACCGCTACGGACCTCCACCA-(TAMRA)-3′	X59733
	F	5′-GGCGAAGCCAGAGGAAACT-3′	
	R	5′-GACGACCGATTGCACGTC-3′	
IL-1β	Probe	5′-(FAM)-CCACACTGCAGCTGGAGGAAGCC-(TAMRA)-3′	AJ245728
	F	5′-GCTCTACATGTCGTGTGTGATGAG-3′	
	R	5′-TGTCGATGTCCCGCATGA-3′	
IL-6	Probe	5′-(FAM)-AGGAGAAATGCCTGACGAAGCTCTCCA-(TAMRA)-3′	AJ250838
	F	5′-GCTCGCCGGCTTCGA-3′	
	R	5′-GGTAGGTCTGAAAGGCGAACAG-3′	
TNF-α	Probe	5′-(FAM)-TGCTGAGAAGGAACAAACTGGTGGT-(TAMRA)-3′	AJ009800
	F	5′-CCCATCCCTGGTCCGTAA-3′	
	R	5′- GGCGGCGTATACGAAGTAAAG-3′	
IL-10	Probe	5′-(FAM)-CGACGATGCGGCGCTGTCA-(TAMRA)-3′	AJ621614
	F	5′-CATGCTGCTGGGCCTGAA-3′	
	R	5′-CGTCTCCTTGATCTGCTTGATG-3′	
IFN-γ	Probe	5′-(FAM)-TGGCCAAGCTCCCGATGAACGA-(TAMRA)-3′	Y07922
	F	5′-GTGAAGAAGGTGAAAGATATATCATGGA-3′	
	R	5′-GCTTTGCGCTGGATTCTCA-3′	

Table 1. Primer and probe sequences

3.2.8. Statistical analysis for qRT-PCR and microbiome analysis

Cytokine mRNA expression for control and treated ceca from days 2 and 4 were quantitated using a method described by Kaiser et al. (50) and Moody et al. (110). Statistical analysis was performed with SAS (version 9.4, Cary, NC, USA) based on the data collected from each trial for the qRT-PCR data. The Shapiro-Wilk's test for normality was used to determine if the fold change within each group was parametric or non-parametric, with an alpha of 0.05. For all analyses, statistical significance was considered if $P \leq 0.05$. All data were found to be non-parametric and were summarized

as median values. An ad-hoc analysis using the Kruskal-Wallis test was conducted to determine where the statistical differences lie between treatments. The ceca samples for the IL-1B, IL-6, IL-10, TNF-α, and IFN-γ were quantified using the 40-C_t method, as outlined by Eldaghayes et al. (108). The results were reported in fold change values.

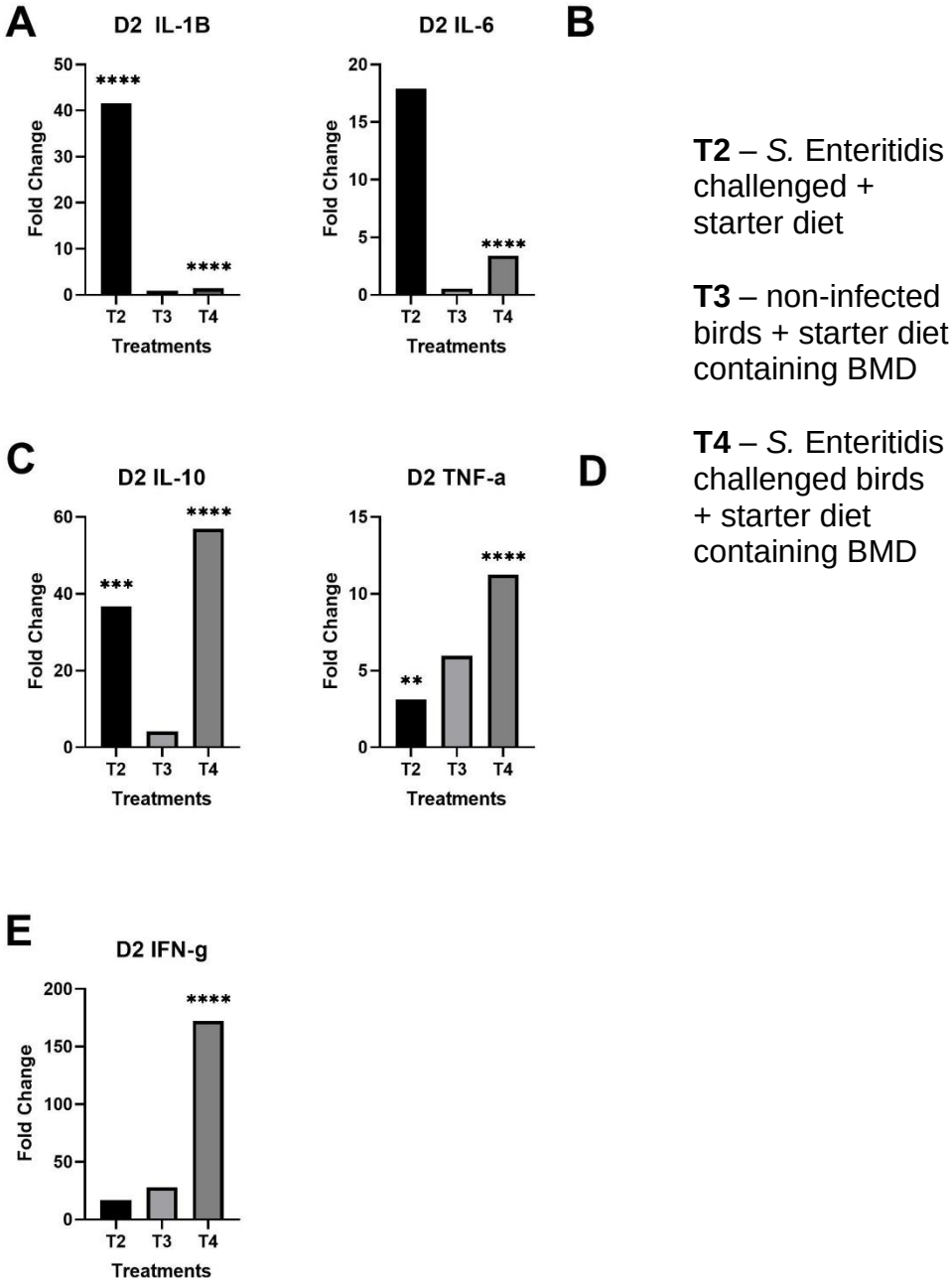
The frequency of bacterial families were submitted to the Kruskal-Wallis test under non-parametric one-way ANOVA and if there were significant differences seen ($P \leq 0.05$), a post-hoc analysis using Dunn's multiple comparison test to separate the means using GraphPad Prism (version 8.0, La Jolla, CA, USA). For the microbiome correlation analysis, Pearson correlation analysis between the mean percentage of bacterial families and the fold change of cytokines was performed using SAS (version 9.4, Cary, NC, USA) with $\alpha \leq 0.05$. The Pearson correlation was carried out within each treatment on day 2 and day 4.

3.3. Results

3.3.1. Real-time quantitative RT-PCR results

All results are reported as averaged triplicate experiments. Overall, there was significant upregulation of proinflammatory and regulatory cytokines in *S.* Enteritidis challenged birds given BMD on day 4 (T4) than non-infected birds given bacitracin (T3), indicating a stimulation of the immune system. Notably, there were statistically significant fold changes on day 2 of IL-1β in T2, IL-6 in T2 and T4, IL-10 in T2 and T4, TNF-α in T2 and T4, and IFN-γ in T4 (**Figure 1**). In day 4, there were statistically significant fold changes of IL-1β in T2 and T4, IL-6 in T4, IL-10 in T2 and T4, TNF-α

in T2 and T4, and IFN-γ in T4. As previously shown, *Salmonella* induces pro-inflammatory response within 2 days of infection and reprogrammed to anti-inflammatory response by day 4. However, the treatment of birds with bacitracin inhibited the reprogramming in the *Salmonella* challenged birds (T4) (**Figure 2**).



T2 – *S.* Enteritidis challenged + starter diet

T3 – non-infected birds + starter diet containing BMD

T4 – *S.* Enteritidis challenged birds + starter diet containing BMD

Figure 1. Fold changes of day 2 cecal samples by treatment per panel
(a) Day 2 fold changes by treatment for IL-1β; (b) Day 2 fold changes by treatment for IL-6; (c) Day 2 fold changes by treatment for IL-10; (d) Day 2 fold changes by treatment for TNF-α; (e) Day 2 fold changes by treatment for IFN-γ. All results are reported as averaged triplicate experiments. The starred bars indicate differing levels of significant p-values. Each treatment (N=42) is compared to T1.

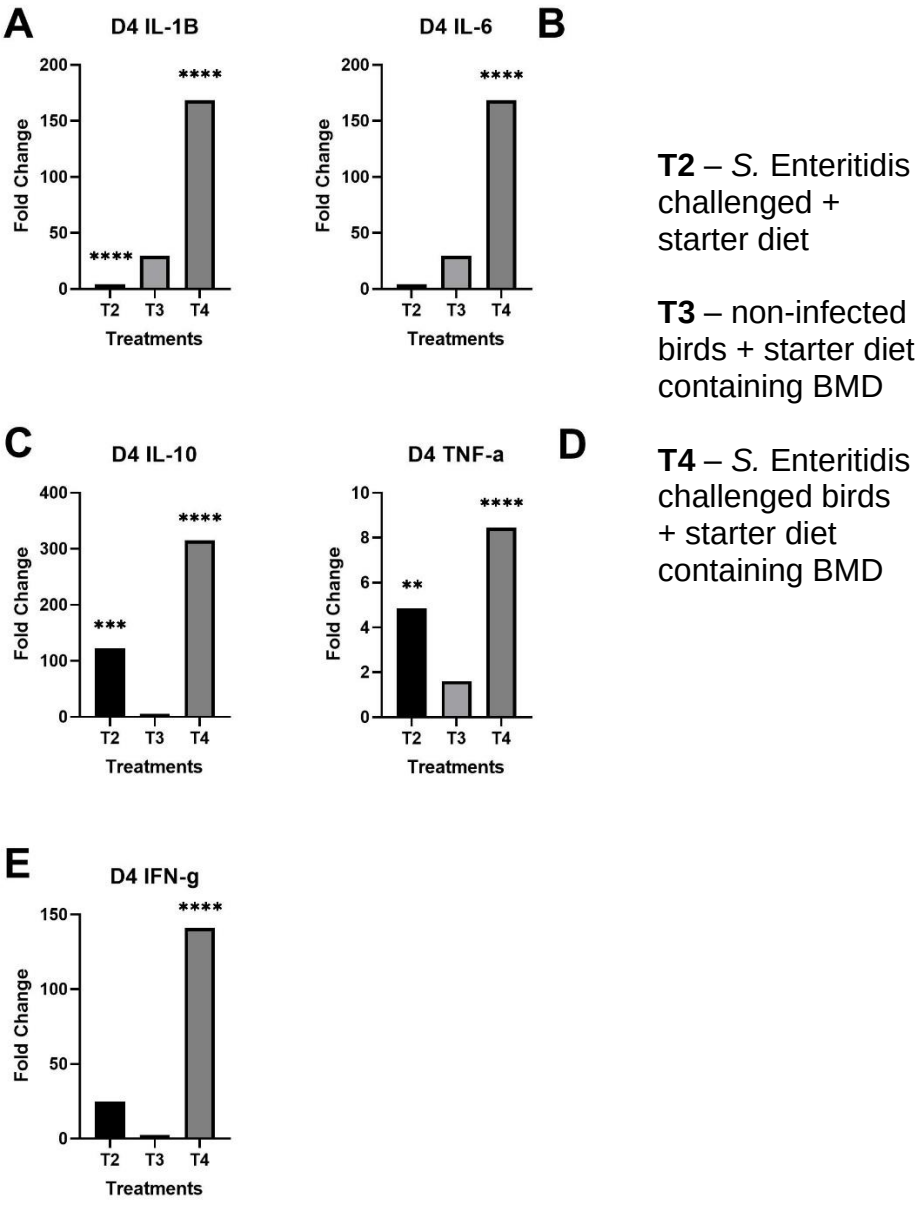


Figure 2. Fold changes of day 4 cecal samples by treatment per panel
(a) Day 4 fold changes by treatment for IL-1β; (b) Day 4 fold changes by treatment for IL-6; (c) Day 4 fold changes by treatment for IL-10; (d) Day 4 fold changes by treatment for TNF-α; (e) Day 4 fold changes by treatment for IFN-γ. All results are reported as averaged triplicate experiments. The starred bars indicate differing levels of significant p-values. Each treatment (N=42) is compared to T1.

3.3.2. Microbiome composition

To understand whether bacitracin-treatment alteration of the cecal microbiota composition could account for the inhibition of immune reprogramming in salmonella-infected birds, the cecal microbiota was evaluated for both days 2 and 4 for all treatment groups. Relative abundances are reported as median values (**Table 2**) and only the high frequency bacterial families are reported. Across all treatments, there was increased diversity of taxa in day 4 compared to day 2. The most abundant bacterial families observed in the ceca were *Enterobacteriaceae* and *Clostridiaceae* during day 2 (**Figure 3a**). The 16S rRNA sequence analysis of the ceca microbiome (day 2) of control birds fed control feed (T1) revealed a microbiome dominated by the family *Clostridiaceae*. Birds infected with *Salmonella* fed control feed (T2) was dominated by *Enterobacteriaceae* (73.6%) followed by lesser presence of *Clostridiaceae* (23.5%). Birds fed bacitracin (T3) had mostly the presence of *Clostridiaceae* (86.5%) followed by a much lesser presence of *Enterobacteriaceae* (4.45%). In the final group with birds infected by *Salmonella* fed a bacitracin feed (T4), there was mostly the presence of *Enterobacteriaceae* (68.1%) followed by *Clostridiaceae* (25%). By day 4 (**Figure 3b**), there was an increased diversity of the microbiome, still dominated by *Clostridiaceae* but with increased presence of *Paenibacillaceae* and *Enterobacteriaceae*. In the treatment group with SE infection fed control feed (T2), there were mostly *Enterobacteriaceae* (>50%) and lesser presence of *Clostridiaceae* in day 2 ceca. By day 4, there were still mostly *Enterobacteriaceae* and *Clostridiaceae* but with increased presence of *Paenibacillaceae*. The control group (T1) was still mostly dominated by

Clostridiaceae (69.1%) but with a marked increase of *Paenibacillaceae* (8.23%) and starting presence of *Bacillaceae* (1.7%). Birds infected with *Salmonella* fed control feed (T2) was observed to have decreased frequency of *Enterobacteriaceae* (58.9%) as compared to day 2, and a starting presence of *Paenibacillaceae* (3.02%) with not much change in the *Clostridiaceae* frequency (23.8%). In the non-infected group fed with bacitracin (T3), the cecal composition has varying changes: large *Clostridiaceae* frequency (40.3%) but with an increased frequency of *Lachnospiraceae* (12.3%) and *Paenibacillaceae* (7.25%). The final group of birds challenged with *Salmonella* fed with bacitracin feed (T4) also had increased frequency of *Lachnospiraceae* (1.25%) and *Paenibacillaceae* (1.98%) but still dominated by *Enterobacteriaceae* (48.5%) and *Clostridiaceae* (34.3%).

When comparing *Salmonella* challenged group (T2) with *Salmonella* challenged with bacitracin in feed group (T4), there is not much difference in diversity and frequency of families on day 2 but there is a notable decrease of *Enterobacteriaceae* and increase of *Lachnospiraceae,* and to a lesser degree, increased frequency of *Clostridiaceae* and decreased frequency of *Paenibacillaceae.* For the less abundant families, there were statistically significant differences within those groups. There was an observed 10.5% decrease in *Clostridiaceae* between T2 and T4 on Day 4 as well as a 10% decrease in *Enterobacteriaceae* (**Table 2**).

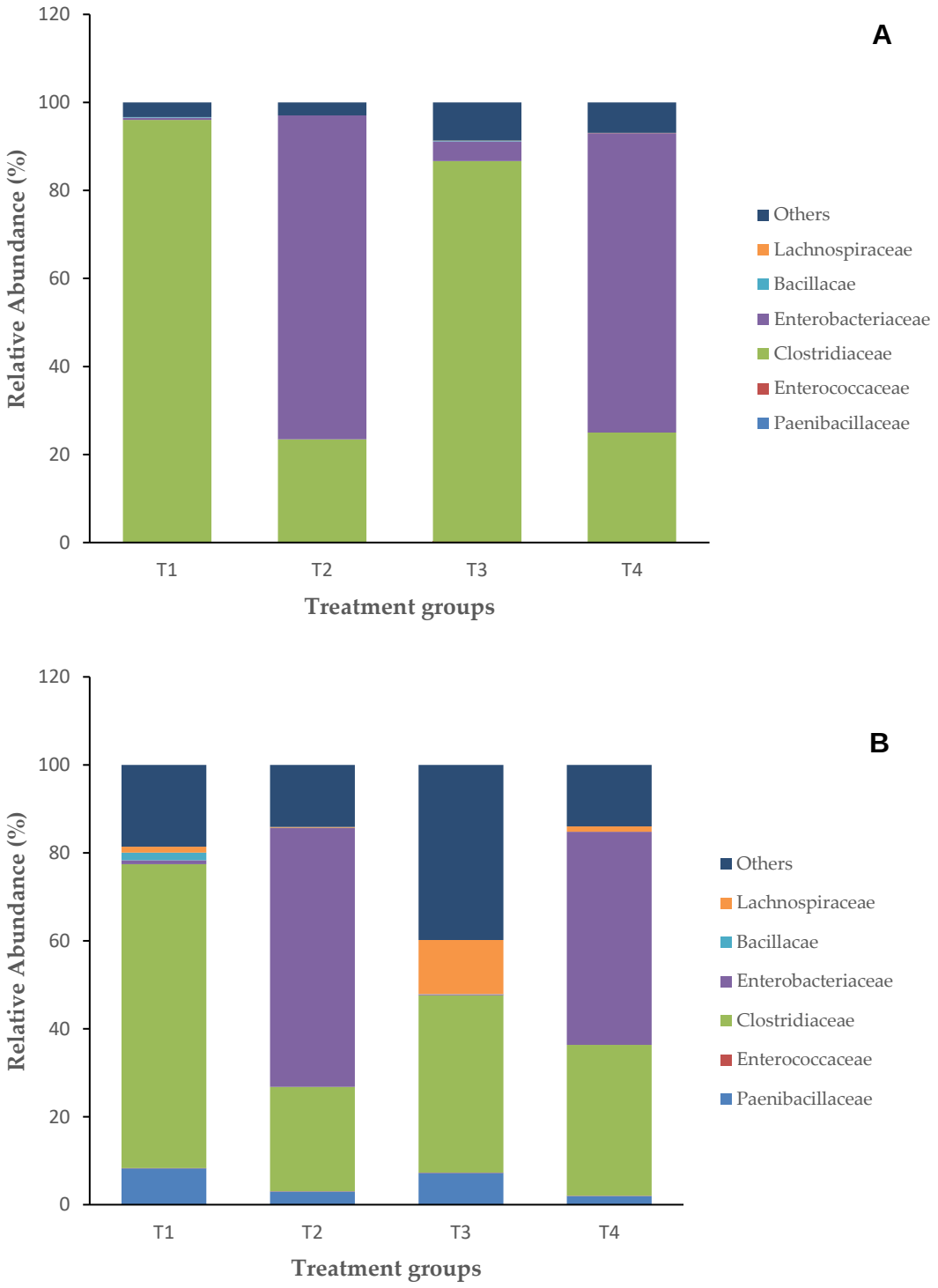


Figure 3. Microbiota composition based on relative abundance: (A) Microbiota composition of day 2 chicken ceca; (B) Microbiota composition of day 4 chicken ceca

Group	Paenibacillaceae		Lachnospiraceae		Clostridiaceae		Enterobacteriaceae		Bacillaceae	
	Day 2	**Day 4**	**Day 2**	**Day 4**	**Day 2**	**Day 4**	**Day 2**	**Day 4**	**Day 2**	**Day 4**
T1	$0.11^{a,b}$	8.23^{a}	0	1.32^{b}	95.9^{a}	69.1^{a}	0.55^{a}	$0.94^{a,c}$	$0.14^{a,c}$	1.70^{a}
T2	$0^{b,d}$	$3.02^{a,b}$	0	0.14^{b}	23.5^{b}	23.8^{b}	73.6^{b}	58.9^{b}	0^{b}	0.02^{b}
T3	$0.18^{a,c}$	$7.25^{a,b}$	0.04	12.33^{a}	86.5^{c}	40.3^{b}	4.45^{c}	0.19^{c}	0.20^{c}	0.07^{b}
T4	0^{d}	1.98^{b}	0	1.25^{b}	25.0^{b}	34.3^{b}	68.1^{b}	48.5^{b}	0^{b}	0^{b}

Table 2. The top listed median relative abundance (by %) of observed families in the ceca of day 2 and 4 birds.

^{a,b,c,d}The differing superscripts indicate varying significant values ($P \leq 0.001$)

3.3.3. Beta diversity index of cecal composition

Unifrac was utilized to compare similarities between bacterial communities in the cecal samples across independent time points (day 2 and day 4). Weighted UniFrac plots showed better separation on day 2 than day 4 where more clustering occurred (**Figures 4a-f**). There were large differences in the weighted UniFrac, but not in the unweighted UniFrac (data not shown) between groups. The ANOSIM of the weighted Unifrac across different ages within each treatment group (**Table 3**) displays large quantitative differences between T2 and control on day 2 and T4 and control on day 2. However, the differences between T2 and control ($R = 0.62$, $p \leq 0.001$) and T4 ($R = 0.59$, $p \leq 0.001$) and control are not as similar by day 4. Overall, there appears to be less diversity when bacitracin is incorporated into the treatments, as seen in comparing T1 vs T3 in the ANOSIM analysis.

Age	Comparison	R[1]	Probability[2]
Day 2	T1 vs T2	0.96	**0.001**
	T1 vs T3	0.1	0.002
	T1 vs T4	0.98	**0.001**
Day 4	T1 vs T2	0.62	**0.001**
	T1 vs T3	0.15	0.002
	T1 vs T4	0.59	**0.001**

Table 3. ANOSIM analysis of weighted Unifrac on different day comparisons within each treatment groups

[1]R is the similarity of comparison: 0 means equally similar, 1 means completely dissimilar.

[2]Significant differences are set at $p \leq 0.001$. Significant differences between treatments are bolded.

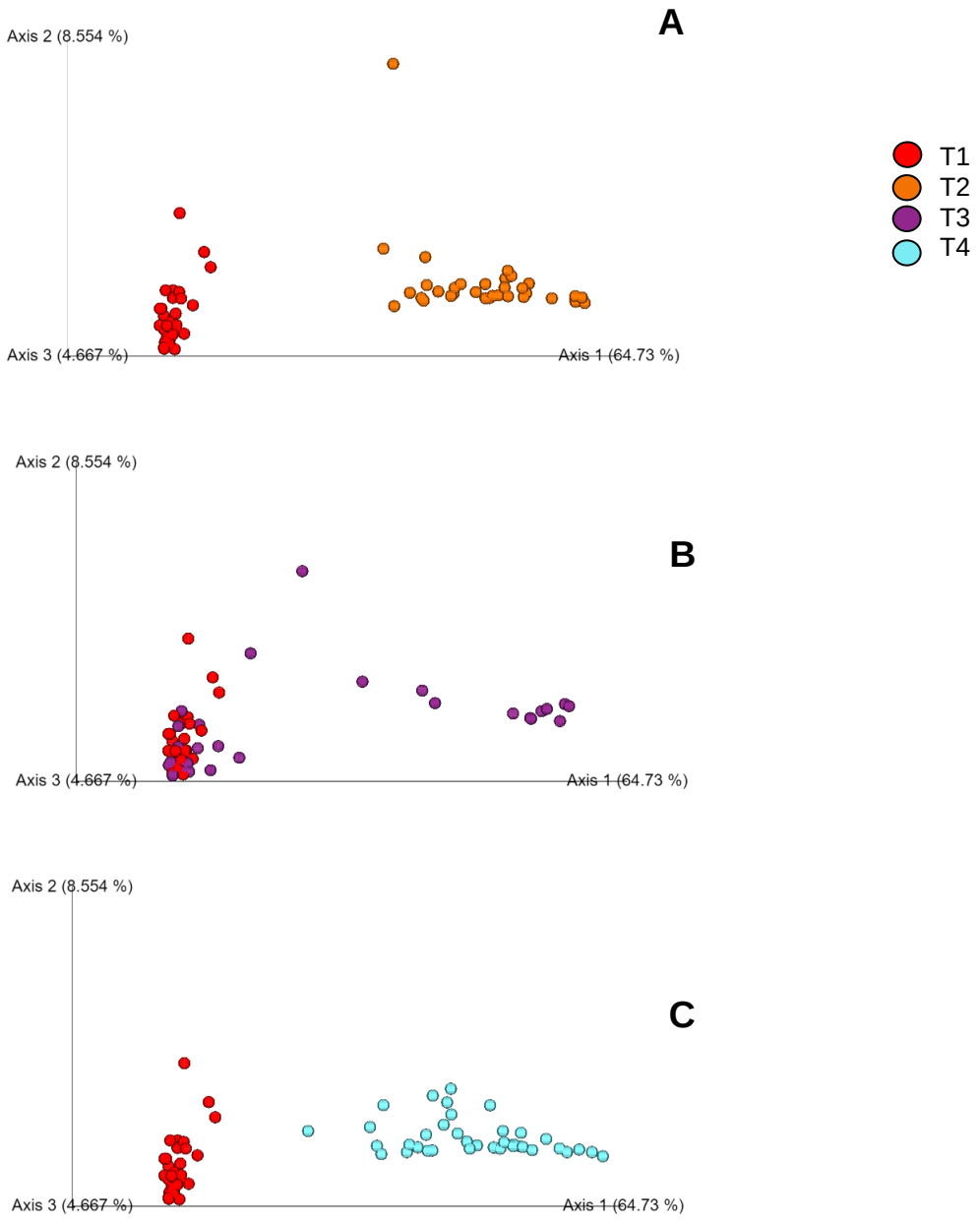


Figure 4. PCoA charts using Unifrac for day 2 and day 4 comparison of treatments (a) d2 T1 vs T2, (b) d2 T1 vs T3, (c) d2 T1 vs T4, (d) d4 T1 vs T2, (e) d4 T1 vs T3, (f) d4 T1 vs T4

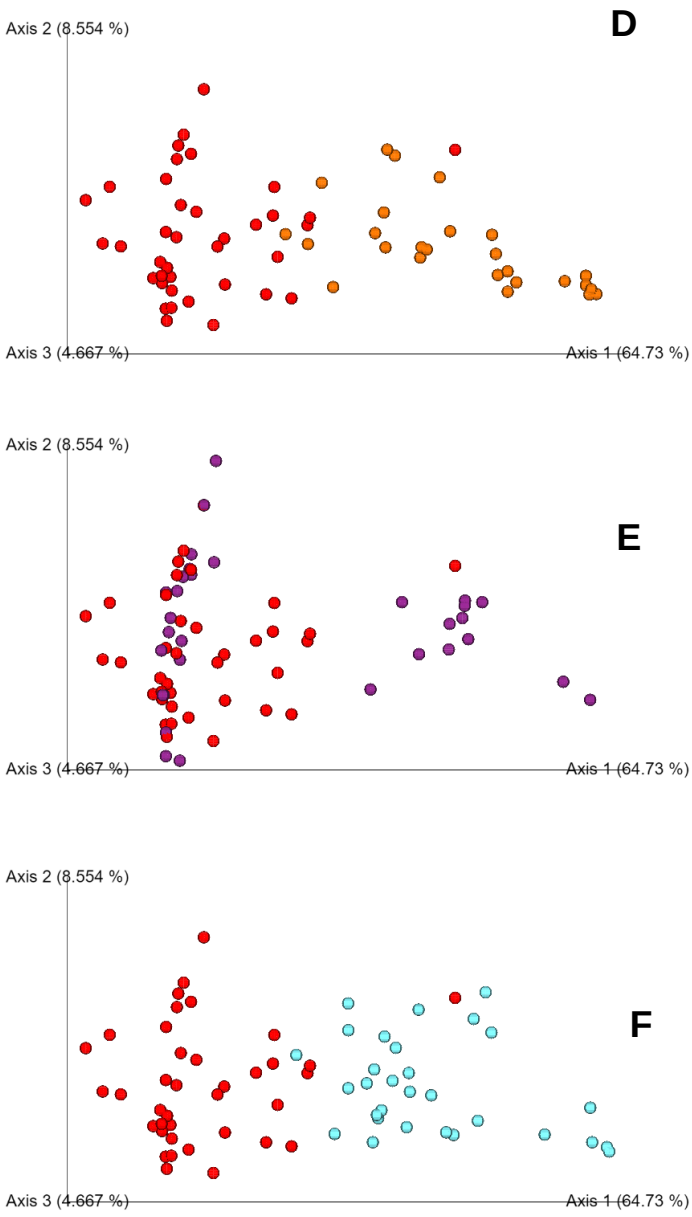


Figure 4 (continued).

3.4. Discussion

As sites of persistent colonization of bacteria, the avian ceca are important sites to study and better understand how the poultry GI tract microbiome interacts with pathogens. Generally, taxonomic diversity increases as the bird ages, enriched mainly with bacteria in the phylum Firmicutes (24). Immediately after hatch, the chicks are exposed to numerous environmental factors that activate their immune system, leading to low-grade inflammation by increased cytokine and chemokine expression (2). Previous studies have already observed bacterial families in the ceca, including as shown in our results *Clostridiaceae*, *Enterococcaceae*, *Streptococcaceae* (111). Our results highlight some key bacterial alterations between the gut bacterial communities and immune function in the crucial first days post-hatch.

Infections with *Salmonella* Enteritidis and antibiotics affected the overall bacterial composition in the ceca, which has also been shown in previous studies with mammalian models as well (25). However, bacitracin did not have much effect on *Salmonella* Enteritidis in this present study, as also seen in the study by Gadde et al. (10) and Mon et al. (5). In fact, it is speculated that AGPs may eliminate some members of *Enterobacteriaceae* but this allows for opportunistic bacteria to proliferate as a result (5). In this study, it appears the addition of bacitracin in the challenge model (T4) modulates the immune system to where the host response was evoked towards the bacitracin and *Salmonella* rather than just the presence of the pathogen. This may explain the mRNA expression data where the IL-1β and IL-6 response in day 2 was inhibited due to the high upregulation of these two cytokines. In previous studies from our group (3,29), the

phenomenon of phenotypic switch occurs between days 2 and 4 in *Salmonella* challenged chicks, going from pro-inflammatory response to an anti-inflammatory, *Salmonella* tolerance response. Infected hosts undergo phenotypic shifts in tissue metabolism, metabolic sensors such as mTOR and AMPK, and immune cell functions (61). The *Salmonella* challenged treatment (T2) follows the previously documented studies: IL-6 and IL-1β are highly upregulated on day 2 and dramatically reduced by day 4 post-infection (61). Therefore, it can be speculated that bacitracin is preventing an overstimulation in the earlier stage, thus allowing the immune system to react later to avoid the tolerance state and to help the immune system to eliminate the bacteria. The strong proinflammatory response, as shown in our mRNA expression data (**Figure 1** and **2**), is stimulated from the host microbiome, not just as a response to the *S.* Enteritidis presence.

According to the UniFrac results, the overall data demonstrated species disappearing or showing up, but with large differences in abundances of those that were already present. As seen in **Figure 4a-f** and **Table 3**, there were notable quantitative differences between T2 and control on day 2 and T4 and control on day 2. However, the differences between T2 and control and T4 and control are not as similar by day 4. Based on the data, there appears to be less diversity when bacitracin is incorporated into the treatments. Juricova et al. (4) concluded that infection with *S.* Enteritidis caused delays in the microbiota development of young chicks, which could explain the phenomena occurring with our results. Interesting, there were differences seen in T2 and T4 compared to the control, but not much between T3 and control. This interaction could also affect the

immune system of the host, adding another layer of variability that has not been well classified in broilers. Oakley and Kogut (24) concluded that Proteobacteria, which includes *Salmonella* spp. had strong correlations to IL-6 pro-inflammatory response. We also observed increased IL-10 expression which can be attributed to the gut modulation via bacitracin and young age of the birds (112). However, the presence of these pro-inflammatory cytokines could also be due to temporal changes in the chicks' unstable gut microbiome (24).

While increased inflammation may affect the animal's performance level, appetite and muscle catabolism, the shifting gut microbiota of young broilers would adjust for the imbalance and lead to less inflammation and energy expenditure for the animal (13,31). The presence of these signaling molecules are important for the epithelial cells at the site of infection and stimulation of macrophages to produce nitric oxide radicals (32). In fact, recent work has demonstrated that antagonists (such as the BMD in this case) allow pathogenic *Enterobacteriaceae* members including *Salmonella* spp. to upregulate the expression of antimicrobial proteins to survive (33). Thus, this disruption of the microbiota increases the pathogen's drive to survive by changing the state of inflammatory response. Furthermore, the presence of *Enterobacteriaceae* has been shown to influence the amount of metabolites expressed in the GIT, especially with short chain fatty acids (SCFAs) (30). SCFAs are important for energy production for epithelial cells and immune cells including T cells and macrophages (34). T3 (bacitracin only) did not seem to produce any statistically significant fold changes when compared to the control across both days. This may be due to the subtherapeutic levels provided, in which previous studies (113,114) also

did not see significant changes in the cecal community structure but did see changes in the community structure overall. We also observed high frequency of *Enterobacteriaceae,* which *Salmonella enterica* is a part of, on both days in *Salmonella* infected birds treated with subtherapeutic bacitracin (T4). This could be due to bacitracin's inhibitory action primarily against Gram-positive bacteria even though it is a broad-spectrum antibiotic. Similarly seen in a study by Kumar et al. (115), this may be occurring due to *Salmonella* outcompeting the resident microflora for nutrients which allows *Enterobacteriaceae* to flourish in the ceca.

The family *Clostridiaceae*, including *Clostridium perfringens,* is of interest in both humans and chickens. On both days tested, the family *Clostridiaceae* persisted, although in varying percentages, since it is generally part of the resident microbiota of the gut (26). In chickens, pathogenic *C. perfringens* causes necrotic enteritis and are usually prevented by AGPs such as bacitracin (26). A previous study by Ballou et al. (116) reports increased cecal diversity of bacterial communities, mainly within the order *Clostridiales,* in which our present study confirms. The high frequency of members of the order *Clostridiales* in the ceca have also been linked to improved growth performance in chickens which is an important factor for the broiler industry to consider (37,117). This family is also closely linked to T_{reg} cells, specifically its ability to increase the frequency of T_{reg} cells (118). The increased presence of Tr_{eg} cells promote the expression of IL-10 to dampen inflammation in the local area (119). T_{reg} cells of *S.* Enteritidis challenged birds had greater expression of IL-10 mRNA than non-infected controls which can also be seen in the present study (T2 and T4) for both time points (120).

Although there was a very low abundance present for *Enterococcaceae* (not shown) in our present study, the high abundance of *Enterococcaceae* and *Enterobacteriaceae* has been linked with dysbiosis due to the involvement of 4-guanidinobutyric acid production (121). Another minor present bacterial family was *Lachnospiraceae,* identified as poultry probiotic bacteria, which appears to remain low in abundance until the chickens are much older in age, but this family of bacteria was one of the most modulated members of the cecal community when bacitracin was provided (**Figure 3a-b**) (37,117). Depending on the genera, this family has been widely associated for the ability to produce beneficial metabolites specifically SCFAs, influence dietary digestion, and involved in metabolic disease regulation for mammalian hosts as well (122). This family has been found in mucosal folds of the GI tract, suggesting their interaction with the lamina propria immune cells make *Lachnospiraceae* an immune regulator to prevent pathogen colonization (123). Furthermore, studies have documented an overall reduction of probiotic-related bacteria in the intestinal tract of chickens with the usage of AGPs (37,124,125). Addition of an SCFA like butyrate has demonstrated to reduce invasion abilities and colonization abilities of *Salmonella* in the host (5,126). Interestingly, the effects of SCFAs tend to produce anti-inflammatory effects on host immunity(35). This study has observed upregulation of proinflammatory cytokines although this could be attributed to the unstable microbiota at young age or interactions with other metabolites.

Overall, our results demonstrate the effects of bacitracin on cecal composition and its interaction with *S.* Enteritidis in young chicks. There is a phenotype change in the ceca due to the shifting microbiota from including bacitracin: the phenotypic

reprogramming has been altered by adding bacitracin. The decrease in *Enterobacteriaceae* and *Clostridiaceae* and the increase of *Lachnospiraceae* indicate the definitive phenotypic change. It has been previously reported that the immunometabolic reprogramming in the cecal tissue occurred during infection with *S. enterica* (3,29). The current study validates this phenotype switch event while also providing initial evidence of a delayed phenotype switch in birds provided subtherapeutic bacitracin while infected with *S.* Enteritidis. While the study looked at a short time frame, the study demonstrated that the shift in microbiota can affect the immune reaction, resulting in the altering downstream energy requirements of the host (31). These results provide insight of the phenotype changes occurring with the cecal bacterial population and its indirect influence on the immune system. It also provides further useful information on what types of beneficial effects that future ATAs should provide for the host at a baseline health and growth promotor level. It is possible that strong correlations were not observed due to the short tested age range and the rapidly changing microbiome is affected more by age than treatment, as also seen by Ballou et al. (116) and Danzeisen et al. (124). Future studies should look at a longer study duration and other segments of the GIT for a wider overview of bacterial communities.

CHAPTER 4: "SUPPLEMENTING CHESTNUT TANNINS IN THE BROILER DIET MEDIATES A METABOLIC PHENOTYPE OF THE CECA"*

4.1. Introduction

By moving away from antibiotics in livestock production, there is a growing interest from the poultry industry to find plant-based alternatives to replace antibiotics in feed. There has been increased interest in utilizing plant-based compounds or phytobiotics as antibiotic growth promoter (AGP) alternatives, including chestnut tannins (*Castanea sativa*) (37,80). Plant-based tannins can be categorized into two major groups: condensed tannins or hydrolyzable tannins (127). Tannins can be found in many plant species, mostly in the inedible portions of the plant such as the bark or wood (17,86). Due to enhanced research, the previous knowledge that tannins possess anti-nutritional effects in livestock species is outdated and the benefits vary on poultry species and dosages in feed (87). The bioactive compounds, polyphenols, found in tannins allow them to be effective immunomodulatory additives that promotes human and animal health (17). While the mechanism of action is still not widely understood, the benefits of phytobiotics in livestock animals include antioxidation properties, stabilization of intestinal microbiome, and improvement of immune system through immunomodulatory effects (21). Extensive studies done in swine exhibit strong

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evidence of phytobiotics as likely alternatives to antibiotics to improve growth performance and health (20,82,83). Ruminant producers have increased interest in growth promoters because of the efficient utilization of energy during rumen fermentation (84). The usage of tannins in the ruminant industry has been of major interest because of the tannins' ability to play a role in rumen protein utilization to improve feed efficiency (128).

Previous studies evaluated the functionality of different tannin species against pathogenic infections across livestock species, showing anti-microbial activity in concentrations ranging between 0.5-1 kg/ton (19,21,83). Importantly, tannins in general have also been shown to improve feed efficiency, growth performance, and intestinal health, as seen when up to 0.2% chestnut tannins (ChT) are added into feed (23,129). However, inclusions of 2% ChT in other avian studies did not improve growth performance and affected palatability. Based on the studies conducted, the usage of tannins in production have shown potential to be efficient as growth promoter alternatives, given the quality and concentration provided (23).

While the previously mentioned studies provide information about microbiological, immunological, and performance data (19,21,93), there is a lack of knowledge about the mechanism of the host metabolic interactions in the intestine, specifically the metabolic reaction to dietary ChT. Tannins exert pro-inflammatory immune responses but for a short-lived period and the anti-inflammatory responses follow soon after (22). Tannins can also exert a synergistic effect with AGP alternatives to promote gut health (23). Currently, there are proposed mechanisms for

immunomodulatory activity based on different types of feed additives (91,130). While gene expression provides important information regarding pathogen interaction, there are disadvantages to genetic approaches including its inability to accurately predict the cellular phenotypes (3).

Studying phosphorylation events provides information on the mechanism of post-translational modification, which offers insight in cellular and tissue phenotypes (68,69). Peptide arrays for kinomic analysis have already been widely utilized across scientific disciplines (69–71). The kinome array provides functional phenotype data, indicating changes within tissue metabolism and immune response to an infection (29). By understanding the kinase activity, there are increased insights into identifying specific biomarkers to provide future therapeutic targets (69). Recent advancements have provided a species-specific peptide array for livestock species, including poultry (69,74). This integrated array demonstrates the importance of combined immunity and metabolic data on the animal's overall health and growth performance (74).

Therefore, the objective of this study was to determine the metabolic phenotype changes affected by ChT in the ceca. By using gene expression and kinome array, we were able to identify global metabolic phosphorylation-based events in the ceca of birds fed ChT at high doses compared to birds fed regular starter diets.

4.2. Materials and Methods

4.2.1. Experimental animals, housing, and treatments

All experiments conducted were in accordance with guidelines set by the United States Department of Agriculture Animal Care and Use Committee (USDA ACUC #2019001), which meets all federal requirements as defined in the Animal Welfare Act, and the Human Care and Use of Laboratory Animals. A total of 200 male day-of-hatch broiler chicks were obtained from a local commercial hatchery and assigned to two treatment groups with two separate experiments, totaling 50 chicks per pen: (1) control feed – normal starter feed (n=50) and (2) 1% ChT inclusion feed (n=50). The 1% ChT inclusion was determined to be most effective without compromising palatability for the birds. Chicks were randomly distributed into each group in pens with fresh pine shavings, water, and starter diet *ad libitum.* All treatments were fed a corn/soybean-based crumble diet, and they differ in AGP or tannins inclusion. The diets were formulated to meet or exceed broilers requirements (**Table 4**). The hydrolyzable chestnut tannin additive (Silvateam s.p.a©., Buenos Aires, Argentina) contained 75% tannin content, supplemented with 94% dry matter, lignin and sugars. The experimental process lasted 10 days per replicate experiment. This study was repeated one more time for a total of two replicate experiments.

Ingredients	%
Corn	59.81
SBM 48%	33.84
Monocalcium phosphate 21	1.56
Soy oil	2.09
Choline chloride	0.10
Limestone	1.56
Salt	0.33
L-lysine HCL	0.19
DL-methionine	0.28
Vitamin premix	0.13
Mineral premix	0.05
L-Threonine	0.05
Calculated nutrients, %	99.99
Protein	22.00
Calcium	0.90
Available phosphorus	0.45
AMEn (Kcal/lb)	1,365.00
Digestible methionine	0.59
Digestible total sulfa amino acid	0.88
Digestible threonine	0.77
Digestible lysine	1.18
Choline	1,256.87
Sodium	0.16
Potassium	0.84
Chloride	0.20

Table 4. Calculated composition of starter diets. The total basal diet contained 1,365 kcal/lb.

4.2.2. Sample collection and processing

On each necropsy day, 10 birds/group were euthanized via cervical dislocation and necropsied on days 2, 4, 6, 8, and 10 of each replicate experiment. Both ceca were removed, flushed with PBS, and flash frozen in liquid nitrogen in order to preserve the

kinase enzymatic activity. The frozen tissues were moved into a -80^{o}C storage freezer until further processing.

4.2.3. Real-time quantitative RT-PCR

The immune portion was quantitated by gene expression studies, specifically through a TaqMan based assay adapted from Eldaghayes et al. (108). Total RNA was extracted using a Qiagen RNeasy® Plus kit (Germantown, MD, USA) and evaluated with a NanoDrop™ 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Ceca stored in RNALater were used for RNA isolation with the Qiagen Rneasy Plus Kit. The ceca were cut longitudinally to expose the lumen and any remaining fecal matter was gently removed with forceps as to not disturb the mucosal layer. For each group, there were 10 ceca processed per replicate experiment for qRT-PCR.

Cytokine mRNA expression levels were ascertained using RT-PCR with 28S as the reference gene. The RNAs were stored at -80^{o}C until plate setup. The cytokines IL-1B, IL-6, IL-8, IL-10, and IFN-γ were quantified utilizing the Eldaghayes et al. method (108). Primer and probe sequences (**Table 5**) for amplification have been described previously by Kogut et al. (109) and Kaiser et al. (50). The plates were run in the Applied Biosystems ABI StepOne Plus PCR system (ThermoFisher Scientific, Waltham, MA, USA) with the previously stated TaqMan Assay under the following conditions: one cycle of 48°C for 30 min, 95°C for 20 s, and 40 cycles of 95°C for 3 s and 60°C for 30 s. Results were calculated with the corrected 40- C_t method, as described in

Eldaghayes et al. (108) and expressed in fold change values. Each sample was run in triplicate for technical replication.

RNA target		*Probe/primer sequence*	*Exon boundary*	*Accession no.*
28S	Probe	5′-(FAM)-AGGACCGCTACGGACCTCCACCA-(TAMRA)-3′		X59733
	F	5′-GGCGAAGCCAGAGGAAACT-3′		
	R	5′-GACGACCGATTGCACGTC-3′		
IL-1β	Probe	5′-(FAM)-CCACACTGCAGCTGGAGGAAGCC-(TAMRA)-3′	5/6	AJ245728
	F	5′-GCTCTACATGTCGTGTGTGATGAG-3′		
	R	5′-TGTCGATGTCCCGCATGA-3′		
IL-6	Probe	5′-(FAM)-AGGAGAAATGCCTGACGAAGCTCTCCA-(TAMRA)-3′	3/4	AJ250838
	F	5′-GCTCGCCGGCTTCGA-3′		
	R	5′-GGTAGGTCTGAAAGGCGAACAG-3′		
IL-8	Probe	5′-(FAM)-CTTTACCAGCGCGTCCTACCTTGCGACA-(TAMRA)-3′	1/2	AJ009800
	F	5′-GCCCTCCTCCTGGTTTCAG-3′		
	R	5′-TGGCACCGCCAGCTCATT-3′		
IL-10	Probe	5′-(FAM)-CGACGATGCGGCGCTGTCA-(TAMRA)-3′	3/4	AJ621614
	F	5′-CATGCTGCTGGGCCTGAA-3′		
	R	5′-CGTCTCCTTGATCTGCTTGATG-3′		
IFN-γ	Probe	5′-(FAM)-TGGCCAAGCTCCCGATGAACGA-(TAMRA)-3′	3/4	Y07922
	F	5′-GTGAAGAAGGTGAAAGATATATCATGGA-3′		
	R	5′-GCTTTGCGCTGGATTCTCA-3′		

Table 5. Real-time quantitative RT-PCR primer and probe sequences
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4.2.4. Statistical analysis for qRT-PCR

Cytokine mRNA expression for control and treated ceca from days 2, 4, 6, 8, and 10 were quantitated using a method described by Kaiser et al. (50) and Moody et al. (110). The 40-C_t values were calculated for each sample with averaged triplicates per sample. Statistical analysis was performed with SAS 9.4 (Cary, NC, USA) based on the

data collected from each trial for the qRT-PCR data. Each time frame had samples comparing 1% ChT-treated birds versus control-fed birds. The Shapiro-Wilks test for normality was used to determine if the fold change within each group was parametric or non-parametric, with an alpha of 0.05. For all analyses, statistical significance was considered if $P \leq 0.05$.

All data were found to be non-parametric and were summarized as median values. An ad-hoc analysis using the Kruskal-Wallis test was conducted to determine where the statistical differences lie between the control and 1% ChT. The ceca samples for the IL-1B, IL-6, IL-8, IL-10, and IFN-γ were quantified using the 40-C_t method, as outlined by Eldaghayes et al. (108). The results were reported in fold change values.

4.2.5. Chicken-specific kinome (peptide) array

For the phenotype readout, a peptide array was utilized to provide tissue immunometabolism information from the host. At three of the time points (days 4, 6 and 10), three whole ceca from three randomly selected birds – stored in -80^{o}C – were defrosted for analysis (27 samples total for all three days). Each sample was weighed to obtain a consistent 40 mg sample for the array. The samples were homogenized by the Omni International Bead Ruptor Elite (Kennesaw, GA, USA) in 100 uL of lysis buffer (20 mM Tris–HCl pH 7.5, 150 mM NaCl,1 mM EDTA, 1 mM ethylene glycol tetraacetic acid (EGTA), 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM Na3VO4, 1 mM NaF, 1 µg/mL leupeptin, 1 g/mL aprotinin and 1 mM phenylmethylsulphonyl fluoride). All products were obtained from Sigma Aldrich (St.

Louis, MO, USA), unless indicated. Following homogenization, the peptide array protocol was carried out as per Jalal et al. (72) with alterations described in Arsenault et al. (3). The resulting tissue lysates were applied onto the PepStar peptide microarrays customized by JPT Peptide Technologies GmbH (Berlin, Germany).

4.2.6. Data analysis: kinome array

Data normalization was performed for the kinome array, based on Li et al. (131) using the PIIKA2 online platform (http://saphire.usask.ca/saphire/piika/index.html), a tool designed for *in silico* analysis of phosphorylation sites (132). The array data were analyzed by conducting variance stabilization normalization, and then performing t-test, clustering and pathway analysis for statistical data. Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis were performed by uploading the statistically significant peptide lists to the Search Tool for the Retrieval of Interacting Genes (STRING) (133).

4.3. Results

4.3.1. Real-time quantitative RT-PCR results

Across both replicate experiments, only IL-6 and IL-10 were found to have statistically significant increases in mRNA expression on days 2 and 6 across all days tested in the experiment. **Figure 5** shows the mRNA expression levels of all tested days (days 2, 4, 6, 8, and 10) for IL-6. The results demonstrated that the expression levels of IL-6 were significantly increased following treatment with 1% ChT on day 2 and day 6

when compared to controls. There were no significant differences when compared to controls on days 4, 8 and 10. **Figure 6** shows the mRNA expression levels of all tested days (day 2, 4, 6, 8, and 10) for IL-10. The results also demonstrated that the expression levels of IL-10 were significantly increased following treatment with 1% ChT on day 2 and day 6. However, there were no significant differences when compared to controls on days 4, 8 and 10.

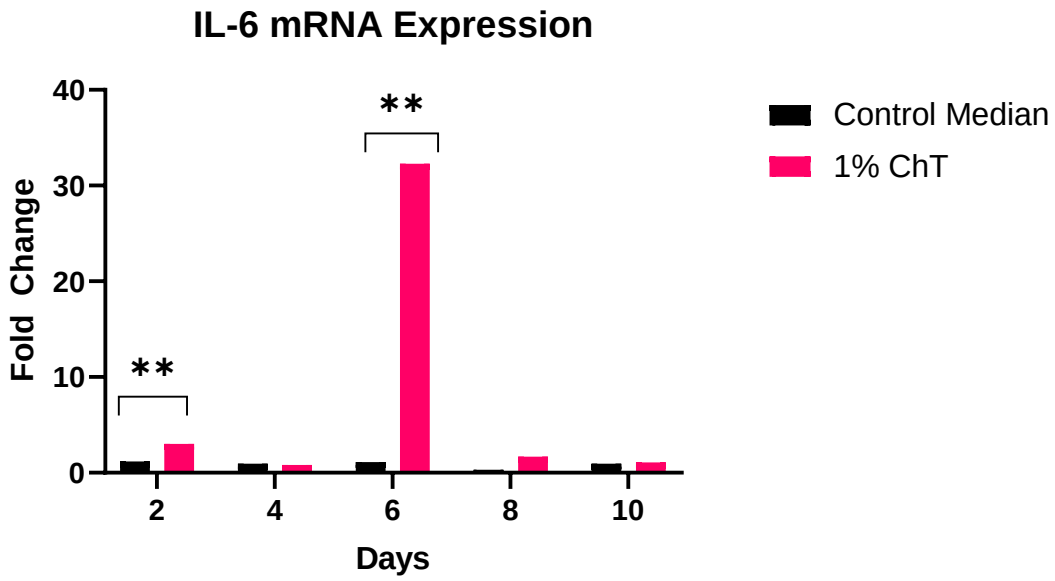

Figure 5. The fold change values based on days tested of the mRNA expression assay for IL-6.
These data reflect the averaged replicate experiments (N=200). The asterisks denote significant differences between the control and 1% ChT group. Reprinted from "Supplementing chestnut tannins in the broiler diet mediates a metabolic phenotype of the ceca"

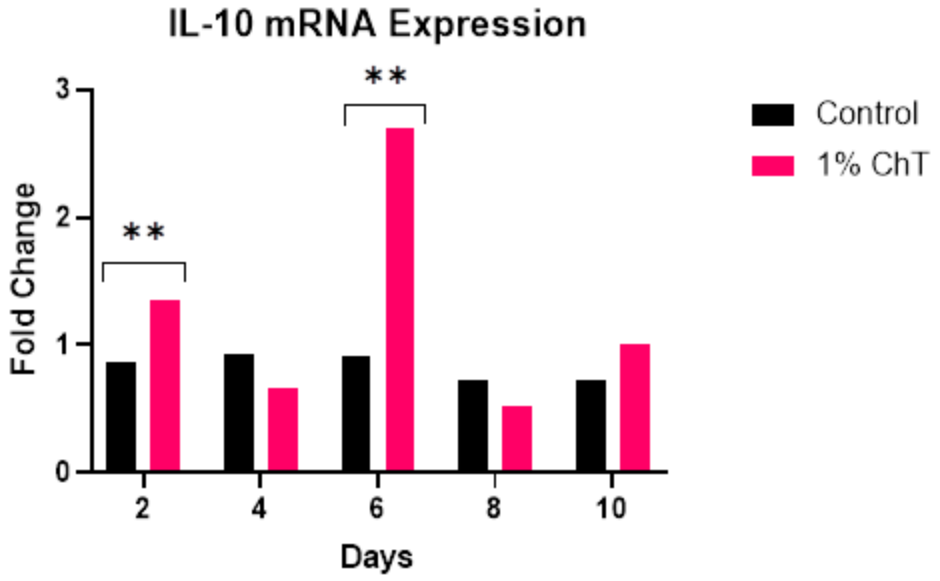

Figure 6. The fold change values based on days tested of the mRNA expression assay for IL-10.
These data reflect the averaged replicate experiments (N=200). The asterisks denote significant differences between the control and 1% ChT group. Reprinted from "Supplementing chestnut tannins in the broiler diet mediates a metabolic phenotype of the ceca"

4.3.2. Kinome results

Using STRING-db (133), the resulting analysis of the kinome data showed distinct metabolic differences between the 1% ChT group compared to the control group. Each day is representative of three birds normalized and combined into representative datasets. The biological process (BP) terms generated from GO for each dataset include sets of molecular events with a defined beginning and end that pertain to the functioning

of the integrated living units (134). Based on the false discovery rate (FDR) of the analysis, values $P \leq 0.01$ were listed and considered statistically significant. The analysis of the kinome data showed distinct differences in the observed BP between 1% CT fed birds compared to the control birds. **Table 6** summarizes the top 15 GO STRING-generated biological pathways of metabolic processes and the number of differentially phosphorylated peptides associated with them. Day 6 samples have the greatest number of primary biological processes related to metabolic pathways out of the three days, indicated by the number of significant peptides. Day 4 had the most amount of fatty acid metabolic process present, but it decreased by day 6 and was not present by day 10.

Additionally, the top metabolic KEGG pathways (135) were obtained from the STRING-db, as summarized in **Table 7**, for 1% ChT-fed group compared to the control group. The pathways of interest were those that showed significant changes at multiple time points. Day 6 samples have the greatest number of altered immune and metabolic pathways out of the three days, indicated by the number of peptides and number of pathways shown in the tables. By day 10, the number of peptides is decreased, as well as the number of pathways.

Biological Processes	Day 4	Day 6	Day 10
phosphate-containing compound metabolic process	100	132	79
primary metabolic process	145	187	122
protein metabolic process	99	136	83
cellular metabolic process	142	184	125
fatty acid metabolic process	90	19	-
glucose metabolic process	11	14	9
glycerolipid metabolic process	18	28	16
cellular lipid metabolic process	37	48	24
glycogen metabolic process	8	9	6
ATP metabolic process	13	11	10
lipid metabolic process	38	50	26
carbohydrate metabolic process	21	25	18
hexose metabolic process	13	15	-
NAD metabolic process	8	11	8
pyruvate metabolic process	10	11	9

Table 6. The top 15 GO metabolic BP identified at days 4, 6, and 10 comparing 1% ChT-fed birds against control birds.

The hyphens indicate non-significant BPs based on the FDR. Reprinted from "Supplementing chestnut tannins in the broiler diet mediates a metabolic phenotype of the ceca"

Metabolic Pathways

	Day 4		Day 6		Day 10	
Identified pathways	**Number of peptides**	**p-value**	**Number of peptides**	**p-value**	**Number of peptides**	**p-value**
PI3K-Akt signaling pathway	34	1.15×10^{-22}	44	4.28×10^{-29}	24	1.11×10^{-13}
MAPK signaling pathway	30	1.75×10^{-20}	49	2.32×10^{-37}	22	2.44×10^{-13}
Metabolic pathway	28	1.39×10^{-05}	32	4.67×10^{-05}	-	-
Insulin signaling pathway	27	5.29×10^{-25}	27	1.30×10^{-22}	20	7.77×10^{-17}
AMPK signaling pathway	21	1.19×10^{-18}	20	7.95×10^{-16}	16	3.16×10^{-13}
HIF-1 signaling pathway	18	1.82×10^{-16}	18	5.60×10^{-15}	12	6.91×10^{-10}
mTOR signaling pathway	18	6.72×10^{-14}	18	2.23×10^{-12}	18	5.53×10^{-14}
cAMP signaling pathway	17	2.85×10^{-11}	13	1.79×10^{-06}	-	-
Glucagon signaling pathway	14	8.32×10^{-12}	15	1.22×10^{-11}	11	9.36×10^{-09}
VEGF signaling pathway	12	7.40×10^{-12}	17	7.06×10^{-17}	-	-
Calcium signaling pathway	11	2.42×10^{-06}	10	0.00012	-	-
Glycolysis/Gluconeogenesis	10	5.39×10^{-09}	10	4.75×10^{-08}	-	-
GnRH signaling pathway	10	4.43×10^{-08}	14	3.03×10^{-11}	-	-
Adipocytokine signaling pathway	-	-	18	3.60×10^{-17}	-	-

Table 7. Summarized table of KEGG metabolic pathways at days 4, 6, and 10 comparing 1% ChT-fed birds and control birds.
The hyphens indicate non-significant BPs based on the p-value. Reprinted from "Supplementing chestnut tannins in the broiler diet mediates a metabolic phenotype of the ceca"

4.4. Discussion

The current study objective was to provide metabolic information on the effects of ChT when incorporated into the broiler feed. Tannins are already known to improve livestock health and growth performance, but the mechanism is still widely unknown

(21). To date, these results are the first of its kind to provide mechanistic information on how host intestine responds to tannins inclusion in the feed. These observed metabolic changes may be related with the improved performance in broilers fed tannins. With the qRT-PCR and kinome data, we were able to provide global post-translational perspectives on how intestine metabolic function is correlated in a tannins-fed diet.

Based on the qRT-PCR data, the cytokines of interest based on our results are the elevated expressions of IL-6 and IL-10 by day 6. As seen in **Figure 5**, the only notable fold changes (values over 2.0) are seen on day 2 and 6 with the 1% ChT group. This shows a significant upregulation of IL-6 and calls for speculation on its role when 1% ChTs are included in the feed. A previous experiment by Ferro et al. (136) concludes that IL-6 may need to work in tandem with other immunomodulatory cytokines (such as IL-1 family cytokines) to stimulate immune responses. In fact, McGeachy et al. (137) discovered that IL-10 is upregulated by IL-6, revealing a dependent relationship between the two cytokines during an immune response. As shown in **Figure 6,** there was significant upregulation of IL-10 only on day 6 in the 1% ChT group, paralleling the IL-6 upregulation trend. This supports what was found in the previously mentioned studies, providing evidence of the IL-10 immunoregulatory properties. As the gene expression results show, the elevated IL-6 upregulation may be the peak time of modulation in the bird (day 6), with IL-10 regulating this pro-inflammatory response. What is important to note is the temporary peak of IL-6 expression: by day 10, the high level of expression is no longer present. This is beneficial for the birds because the chestnut tannins stimulate a response to prime the birds' immunity. While IL-6 is known to have pro- and anti-

inflammatory properties based on the stimulus (136), long-lasting IL-6 presence may contribute to chronic inflammation and tissue damage, which is undesirable for the birds and producers (138). There is also strong evidence of IL-6 involved in metabolic function, rather than just immune response. Flint et al. (139) found that IL-6 induction stimulated a metabolic reprogramming which directly induced an immune suppression in mice. They predicted that this metabolic pathway of IL-6 helps prevent immunological damage due to inflammation. Reviews by Pavlov and Tracey (140) and Ghanemi and St Amand (141) outline further proof of IL-6 and its direct linkage to metabolic pathways, especially affecting insulin pathways and fatty acid synthesis. These reviews discuss how IL-6 should be considered beyond its immunological function due to its effects on lipids, protein, and glucose metabolism.

The current study is the first to demonstrate metabolic importance associated with ChTs, as indicated by the number of post-translational modifications and significant p-values. Based on its known antimicrobial effects, ChTs and other phytobiotics make ideal candidates for growth promotion and health improvements based its ability to retain bacterial diversity in the gut but reducing drug-resistant bacteria (142). Chestnut tannins have already been known to affect bifidobacteria in the ceca of mammals and chickens (37), which have been shown to alter carbohydrate metabolism and other metabolic processes downstream in the host by modifying enzymes and sugar transport pathways (143). This research supports this through the direct alteration of the carbohydrate and primary metabolic pathways (**Table 6**). The main difference in bacterial taxa between control and tannins-fed birds was the increase in bacterial

diversity over time within the tannins-fed group, especially with *Lactobacillus* and *Enterococcus* species (37). These bacterial species utilize sugar metabolism (such as hexose and glucose metabolism) and carbohydrate metabolism which supports our findings in our tannins-fed group (144). Another study found older birds treated with tannins consistently had increased populations of order Clostridiales and family Ruminococcaceae, which have been of interest in the poultry industry as potential probiotic options (37). This is noteworthy evidence of how tannins-fed chickens could have increased members of the family Ruminococcaceae to alter the SCFAs profile in the cecum towards butyrate production. The increased presence of these butyrate-producing communities would indicate the usage of carbohydrate metabolism, fatty acid metabolism, glycerolipid metabolism downstream to ultimately benefit host physiology (144,145).

An interesting note by Diaz Carrasco et al. (37) was the sensitivity effect that dietary tannins had on Gram-positive bacteria, as also noted in rat studies with increased Gram-negative bacteria in the GI tract (146). Molino et al. (147) have shown preliminary data on the importance of tannins in gut microbial fermentation and the nutritional importance in human application. This study provides additional new information on affected pathways in the intestine with the introduction of ChTs in early broiler growth. Although the microbiota of young birds are more prone to fluctuating gut microbial communities, these results support the relevance of chestnut tannins in the feed to not only promote growth but to also provide more evidence as an alternative to antibiotics. Future studies will look at growth promotion effects in depth by investigating the

individual pathways that were altered with the introduction of ChT in the feed. Another future study will also test the other treatment groups (0.2%, 0.08% and 0.03% ChT inclusion groups) for metabolic differences.

Therefore, with the current trend of removing antibiotics in feed, tannins are one of the promising feed additives due to its ability to stimulate immunoregulatory effects and host modulation to prevent resistance, which is ideal as an alternative to antibiotics. This aims to reduce the immune response while redirecting energy towards growth (74). Ideal alternatives would promote health without risking loss of growth promotion or antibiotic resistance (21). Chestnut tannins have already shown applicable results in reducing incidence of necrotic enteritis and *Salmonella* in livestock, although the effects on growth improvements varied based on dosages (19,37,148). The results from this current study are providing further proof to what is currently in the literature of ChTs as a potential alternative to antibiotics. The strong metabolic connection found in our study shows the promising nature of utilizing ChTs as an antibiotic alternative because there would be promotion of growth on top of promotion of health. Previous studies have already observed the host-pathogen interactions of chickens using the kinome array (58,76,149), which shows the importance of viewing immunity and metabolism comprehensively instead of as individual units. With the increasing population, there will be a growing demand for poultry products especially cost-effective feed additives (21). Therefore, identifying an alternative to AGPs will be crucial in the future to keep up with increased demands for safe poultry products. Tannins might be a promising

alternative to AGPs due to the improvements in performance and microbiota, as seen in previous studies, and the current data support its metabolic modulation in the intestine.

In conclusion, it was observed that the influence of ChT in the diet alters gut immunometabolism of broilers. By focusing on the ChT-fed group compared to the control group, the results from these two separate trials demonstrated the metabolic outcome of when ChTs are introduced into the diet. These are the first data in the literature demonstrating pathway data that supports growth and health promotion with ChT. The objective of this study was to provide a global overview of chestnut tannins' effect on the metabolism, providing mechanism information that is currently lacking in the literature. These data offer ChT not only as an immunoregulator but also a potential host-directed therapy option in disease studies. Phytobiotics can offer promising results since they can target metabolic and immunological pathways of the host and may affect the pathogen. With the growing restriction of antibiotics in the feed, this study offers further evidence of ChT as an important alternative to antibiotics.

CHAPTER 5: ROLE OF JAK-STAT PATHWAY IN CHICKS FED WITH CHESTNUT TANNINS

5.1. Introduction

Antibiotic growth promoters (AGPs) were originally incorporated into livestock feed to promote better health and performance. However, with the ban of antibiotics in feed, there is a need for alternatives to antibiotics that will stimulate similar outcomes of AGPs. There has been increased interest in utilizing plant-based compounds or phytobiotics as antibiotic growth promoter alternatives, including chestnut tannins (ChT - *Castanea sativa*) (80,150). Phytogenic compounds are viable candidates to replace AGPs due to bioactive properties that emulate similar properties of antibiotics without fear of antimicrobial resistance (37). Plant-based tannins can be categorized into two major groups: condensed tannins or hydrolyzable tannins (127). Tannins can be found in many plant species, mostly in the inedible portions of the plant such as the bark or wood (17,86). This popular alternative to antibiotic (ATA) is already widely utilized on commercial farms for its overall health benefit and improved performance although the mechanism of how it works is still not fully understood. Host gut immunity are better protected from pathogens via increased villus height:crypt depth ratio, improved intestinal mucosa to prevent tissue damage, affected production of pro-inflammatory and anti-inflammatory cytokines, and enhanced expression of tight junction proteins to modulate the immune system (37,151–153).

Studying phosphorylation events provides information on the mechanism of post-translational modification, which offers insight in cellular and tissue phenotypes (68,69). The species-specific kinome array utilized for this study can measure the kinase activity on the tissue level, therefore, allowing the observation of post-translational modification and further insight on metabolic and immune function (72). Our previous manuscript analyzed the metabolic phosphorylation events when ChTs were included in the diet. Therefore, the objective of this study was to analyze the outcome of specific immune phosphorylation events when broiler chicks are fed a ChT inclusion diet at young age. We analyzed multiple timepoints of chicken-specific kinomic immune changes in avian cecal tissue of chicks fed 1% chestnut tannins. Using this technique, we were able to identify specific phosphorylation-based immune post-translational signaling changes.

5.2. Materials and Methods

5.2.1. Experimental animals, housing, and treatments

All experiments conducted were in accordance with guidelines set by the United States Department of Agriculture Animal Care and Use Committee (USDA ACUC #2019001), which meets all federal requirements as defined in the Animal Welfare Act, and the Care and Use of Laboratory Animals. A total of 200 male day-of-hatch broiler chicks were obtained from a local commercial hatchery and assigned to two treatment groups, totaling 50 chicks per pen per experiment. The treatments were as follows: (1) control feed – normal starter feed (n=50) and (2) 1% ChT inclusion feed (n=50). Chicks were randomly distributed into each group in pens with fresh pine shavings, water, and

starter diet *ad libitum.* All treatments were fed a corn/soybean-based crumble diet, and they differ in AGP or tannins inclusion. The diets were formulated to meet or exceed broilers requirements. The hydrolyzable chestnut tannin additive (Silvateam s.p.a©., Buenos Aires, Argentina) contained 75% tannin content, supplemented with 94% dry matter, lignin and sugars. Each separate experimental process lasted 10 days.

5.2.2. Sample collection and processing

On each necropsy day, 10 birds/group were euthanized via cervical dislocation and necropsied on days 2, 4, 6, 8, and 10 of each separate experiment. Both ceca were removed, flushed with PBS, and flash frozen in liquid nitrogen to preserve the kinase enzymatic activity. The frozen tissues were stored in -80^{o}C until further processing. Ceca stored in RNALater were used for RNA isolation with the Qiagen Rneasy Plus Kit. The ceca were cut longitudinally to expose the lumen and any remaining fecal matter was gently removed with forceps.

5.2.3. Chicken-specific kinome (peptide) array

For the phenotype readout, a peptide array was utilized to provide tissue immunometabolism information from the host. At three of the time points (days 4, 6 and 10), three whole ceca from three randomly selected birds – stored in -80^{o}C – were defrosted for analysis. Each distal end of the cecum was weighed to obtain a consistent 40 mg sample for the array. The samples were homogenized by the Omni International Bead Ruptor Elite (Kennesaw, GA, USA) in 100 uL of lysis buffer (20 mM Tris–HCl

pH 7.5, 150 mM NaCl,1 mM EDTA, 1 mM ethylene glycol tetraacetic acid (EGTA), 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM Na3VO4, 1 mM NaF, 1 μg/mL leupeptin, 1 g/mL aprotinin and 1 mM phenylmethylsulphonyl fluoride). All products were obtained from Sigma Aldrich (St. Louis, MO, USA), unless indicated. Following homogenization, the peptide array protocol was carried out with previously described alterations (72,154). The resulting tissue lysates were applied onto the PepStar peptide microarrays customized by JPT Peptide Technologies GmbH (Berlin, Germany).

5.2.4. Real-time quantitative RT-PCR assay

Total RNA was extracted using a Qiagen RNeasy® Plus kit (Germantown, MD, USA) and evaluated with a NanoDrop™ 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). For each group, there were 10 ceca processed per experiment for qRT-PCR. Cytokine mRNA expression levels were ascertained using RT-PCR TaqMan based assay with 28S as the reference gene. The RNAs were stored at -80ºC until plate setup. The cytokines IL-1B, IL-6, IL-8, IL-17, and IFN-γ were quantified utilizing a previously published method (108). Primer and probe sequences for amplification have been previously published and described (50,109). The plates were run in the Applied Biosystems ABI StepOne Plus PCR system (ThermoFisher Scientific, Waltham, MA, USA) with the previously stated TaqMan Assay under the following conditions: one cycle of 48°C for 30 min, 95°C for 20 s, and 40 cycles of 95°C for 3 s and 60°C for 30 s. Results were calculated with the corrected 40- C_t method (108), and

expressed in fold change values. Each sample was run in triplicate for technical replication.

5.2.5. Data analysis: kinome array

Data normalization was performed for the kinome array using the PIIKA2 online platform (http://saphire.usask.ca/saphire/piika/index.html), a tool designed for *in silico* analysis of phosphorylation sites (131,132). The array data were analyzed by conducting variance stabilization normalization, and then performing t-test, clustering and pathway analysis for statistical data. Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis were performed by uploading the statistically significant peptide lists to the Search Tool for the Retrieval of Interacting Genes (STRING)(133). The pathways are filtered by the minimum required interaction score with high confidence (0.700), in which the thickness of the connection lines indicate the strength of the data support.

5.3. Results

As has been reported previously, broiler chicks fed 1% ChT in the diet had upregulated proinflammatory cytokine, specifically IL-6, mRNA expression in the ceca (155). **Figure 7a-b** depict each separate experimental mRNA expression fold change values across different days tested for the kinome array. The other tested pro-inflammatory cytokine results (IL-1β, IL-8, IL-17, IFN-γ) can be found in **Figure 9a-c**. Out of the proinflammatory cytokines tested, only IL-6 was found to be statistically

significant in fold change differences (more than 32-fold) on day 6 and day 10 in birds fed 1% ChT compared to the controls. On day 4, none of the cytokines tested was considered significant in fold change. By day 10, IL-6 still had a statistically significant 3-fold change compared to the control but not as elevated as it was on day 6.

For the kinome array, one of the major phosphorylated pathways affected by chestnut tannins identified was the JAK-STAT signaling pathway for all days tested. **Figure 8a-c** display the complete interaction network of unique proteins from the kinome array for days 4, 6, and 10. The clustered peptides indicate closely related connections. These figures also reveal the number of links between immune pathways (ones without links are not confidently linked as interactions according to STRING-db). For each day tested, the JAK-STAT pathway was listed as one of the top altered immune pathways. To further evaluate the phosphorylation events, the peptides involved directly or indirectly in the JAK-STAT pathways are listed in **Table 8**. JAK1 was significantly phosphorylated only on day 6 with no change in phosphorylation on days 4 and 10 (as indicated by the hyphen). JAK2 appeared to be a target for dephosphorylation, as seen by decreased phosphorylation at days 4 and 6 in 1% ChT fed chicks compared to the control. Another peptide of interest is the IL6ST peptide (Interleukin-6 receptor subunit beta) which was phosphorylated on days 4 and 10 but interestingly dephosphorylated on day 6. The array also identified STAT1, 3, and 5B activities: significantly increased STAT1 and STAT3 phosphorylation on all three time points, while STAT5B had significantly decreased phosphorylation on day 4 but increased phosphorylation on days 6 and 10. Therefore, the increased presence of JAK and STAT peptides indicate a

stimulation in increased IL-6 to stimulate JAK-STAT signaling pathway due to the inclusion of 1% ChT in the diet.

The other majorly affected immune pathways identified on the kinome array were T cell receptor signaling pathway and chemokine signaling pathway. The resulting phosphorylation events within these pathways are listed in **Tables 9 and 10**. For the chemokine signaling pathway, CCR2 is one of the starting peptides of the pathway. This peptide was only significantly increased in phosphorylation on day 4 but no changes seen on days 6 and 10. Overall, neither the T cell receptor nor the chemokine pathway showed significant activation by feeding 1% ChT to broilers (**Tables 9 and 10**).

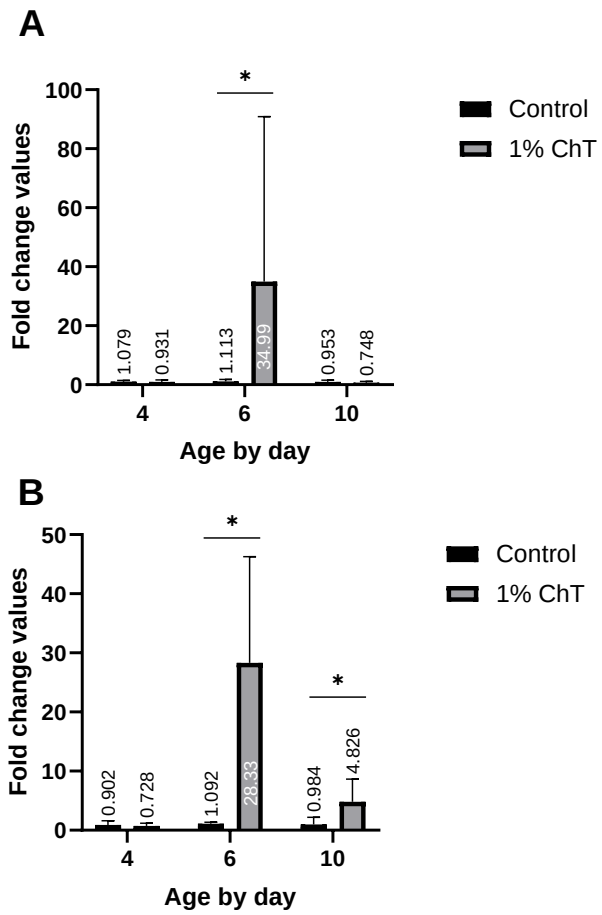

Figure 7. IL-6 mRNA expression of cecal tissue from experimental chicks fed 1% ChT inclusion diet, determined by quantitative RT-PCR:
The days displayed are in correlation to the days tested for the kinome array: (A) 1st experimental fold changes by age tested comparing averaged 1% ChT inclusion group with control group; (B) 2nd experimental fold changes by age tested comparing averaged 1% ChT inclusion group with control group. * = significantly different from the controls ($p \leq 0.05$).

JAK-STAT Signaling Pathway						
	Days of Necropsy					
	4		6		10	
Peptides	Fold change	*p*-value	Fold change	*p*-value	Fold change	*p*-value
AKT3	-1.046	<0.01	-1.041	0.026	-1.023	0.034
CCND1	-	-	1.071	0.015	-	-
EGFR	-	-	1.032	0.041	-	-
EP300	-	-	-	-	1.052	0.031
GRB2	-	-	-1.063	0.02	1.044	0.017
IL12B	-	-	-1.048	0.03	-	-
IL6ST	1.058	0.026	-1.039	0.03	1.041	0.039
JAK1	-	-	1.098	<0.01	-	-
JAK2	-1.059	0.027	-1.042	<0.01	-	-
MTOR	1.061	0.042	-1.058	0.01	-1.041	0.036
PDGFRA	-	-	1.127	<0.01	-1.064	<0.01
PDGFRB	-1.115	<0.01	1.091	0.019	-1.061	0.046
PIK3CB	1.086	<0.01	-	-	-1.049	0.022
PIK3R1	1.058	0.028	-1.025	0.045	-1.076	<0.01
PIK3R2	1.052	0.038	-1.039	0.04	-	-
PIM1	-	-	-1.046	<0.01	-1.043	0.036
RAF1	1.053	0.03	-1.048	0.048	-	-
SOCS3	-1.069	<0.01	-1.052	<0.01	-	-
SOS1	-	-	-	-	1.036	0.029
STAM2	-	-	-	-	-1.057	0.017
STAT1	1.057	0.048	1.060	0.033	1.034	0.034
STAT3	1.067	0.015	1.078	0.031	1.046	0.044
STAT5B	-1.071	<0.01	1.061	<0.01	1.087	<0.01

Table 8. Peptides from the JAK-STAT signaling pathway that displayed statistically significant change in phosphorylation.

Positive values indicate phosphorylation events; negative values indicate dephosphorylation events. Only peptides that displayed a p-value of less than 0.05 are shown**.**

Chemokine signaling pathway			
Peptide	**Day 4**	**Day 6**	**Day 10**
AKT3	↓	↓	↓
ARRB1	↓	↓	-
ARRB2	-	↓	-
CHUK	-	↑	-
CRK	-	-	↑
CRKL	↓	-	-
CRR2	↑	-	-
GRB2	-	↓	↑
GRK5	↓	↓	-
GSK3A	↓	↑	↓
GSK3B	↓	↑	↑
JAK2	↓	↓	-
LYN	-	↑	-
MAP2K1	↑	-	-
NFKB1	-	↑	-
NFKBIA	-	↑	↓
PAK1	↓	-	-
PIK3R1	↑	↓	↓
PIK3R2	↑	↓	-
PRKCD	↓	↓	-
PTK2	↑	↑	↓
PTK2B	↑	↑	↑
PXN	↓	↑	↓
RAF1	↑	↓	-
SHC1	-	↑	-
SHC3	-	↑	-
SRC	-	↓	↑
STAT1	↑	↑	↑
STAT3	↑	↑	↑
STAT5B	↓	↑	↑

↑ = significantly ($P \leq 0.05$) phosphorylated from non-infected control; ↓ = significantly ($P \leq 0.05$) dephosphorylated from non-infected control; - = not significant from non-infected control.

Table 9. Chemokine signaling pathway in chicken ceca on days 4, 6, and 10 of age.

T cell receptor signaling pathway			
Peptide	**Day 4**	**Day 6**	**Day 10**
AKT3	↓	↓	↓
CHUK	-	↓	-
FOS	-	↑	-
FYN	↓	↓	-
GRB2	-	↓	↑
GSK3B	↓	↑	↑
JUN	↓	↓	-
LCK	-	↑	-
MAP2K1	↑	-	-
MAP2K2	-	↓	↑
MAP3K7	↓	↑	↑
NFATC1	↑	-	-
NFATC2	-	↓	-
NFATC3	↑	↓	↑
NFKB1	-	↑	-
NFKBIA	-	↑	↓
PAK1/2	↓	↓	-
PDPK1	↓	↓	-
PIK3R1	↑	↓	↓
PIK3R2	↑	↓	↓
PTPRC	-	↓	-
RAF1	↑	↓	-
ZAP70	-	↓	↓

↑ = significantly ($P \leq 0.05$) phosphorylated from non-infected control; ↓ = significantly ($P \leq 0.05$) dephosphorylated from non-infected control; - = not significant from non-infected control.

Table 10. T cell receptor signaling pathway in chicken ceca on days 4, 6, and 10 of age.

5.4. Discussion

Demands for alternatives to antibiotics, such as ChTs, in livestock feed have increased globally in the past several years. Growing research is showing the importance of the gastrointestinal system and its role in host immune modulation(6,36,156). Current reviews have already been published regarding the evidence of ChTs in improving

overall host health and immunity during pathogenic events but, there is relatively little known about the functional pathways that ChTs play in immunity in a non-infection model. One of the most altered peptide pathways from the present study is the JAK-STAT signaling pathway, which is a crucial pathway for growth, energy, immunity, and overall development of the animal. This pathway is also important for its connection to IL-6 regulation, which was heavily upregulated on day 6 of the mRNA expression array (**Figure 7a-b**). **Figure 8a-c** display the complete interaction network of unique proteins from the kinome array for days 4, 6, and 10. This network reveals the strong patterns towards immune connections, especially with the JAK-STAT signaling pathway (affiliated peptides indicated in the purple circles on each time point). Based on our findings, the kinomic analysis demonstrates the importance of a phytobiotic incorporation in feed, providing further evidence of its potential as a key ATA. These results can also provide useful information regarding how the JAK-STAT pathway gets modified with the inclusion of phytobiotics in livestock diet.

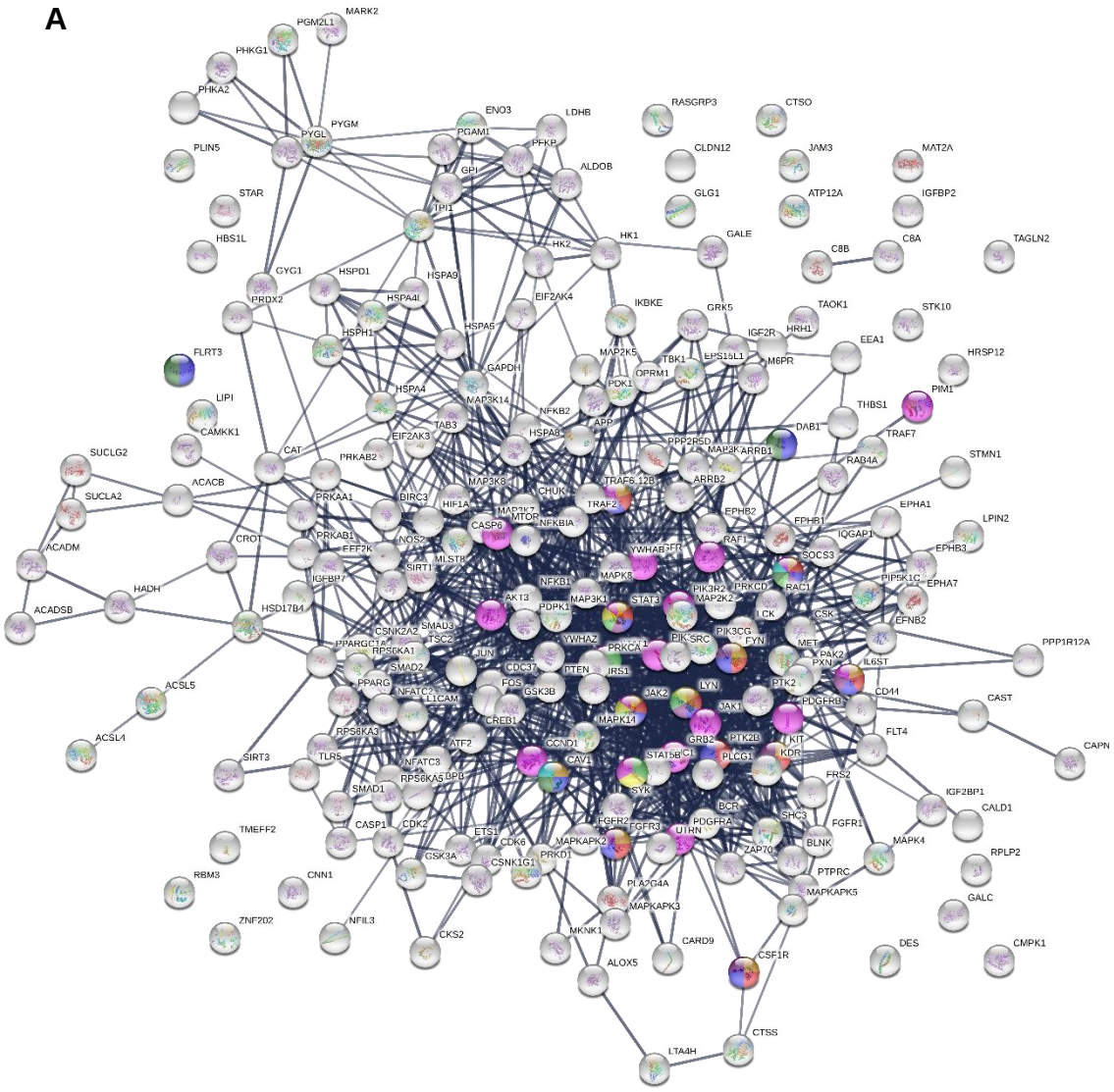

Figure 8. mRNA expression of cecal mRNA from experimental chicks fed 1% ChT inclusion diet, determined by quantitative RT-PCR.

(A) Day 4 fold changes by cytokines tested comparing averaged 1% ChT inclusion group with control group; (B) Day 6 fold changes by cytokines tested comparing averaged 1% ChT inclusion group with control group; (C) Day 10 fold changes by cytokines tested comparing averaged 1% ChT inclusion group with control group. Data represent the median values from two separate experiments. * = significantly different from the controls ($p \leq 0.05$).

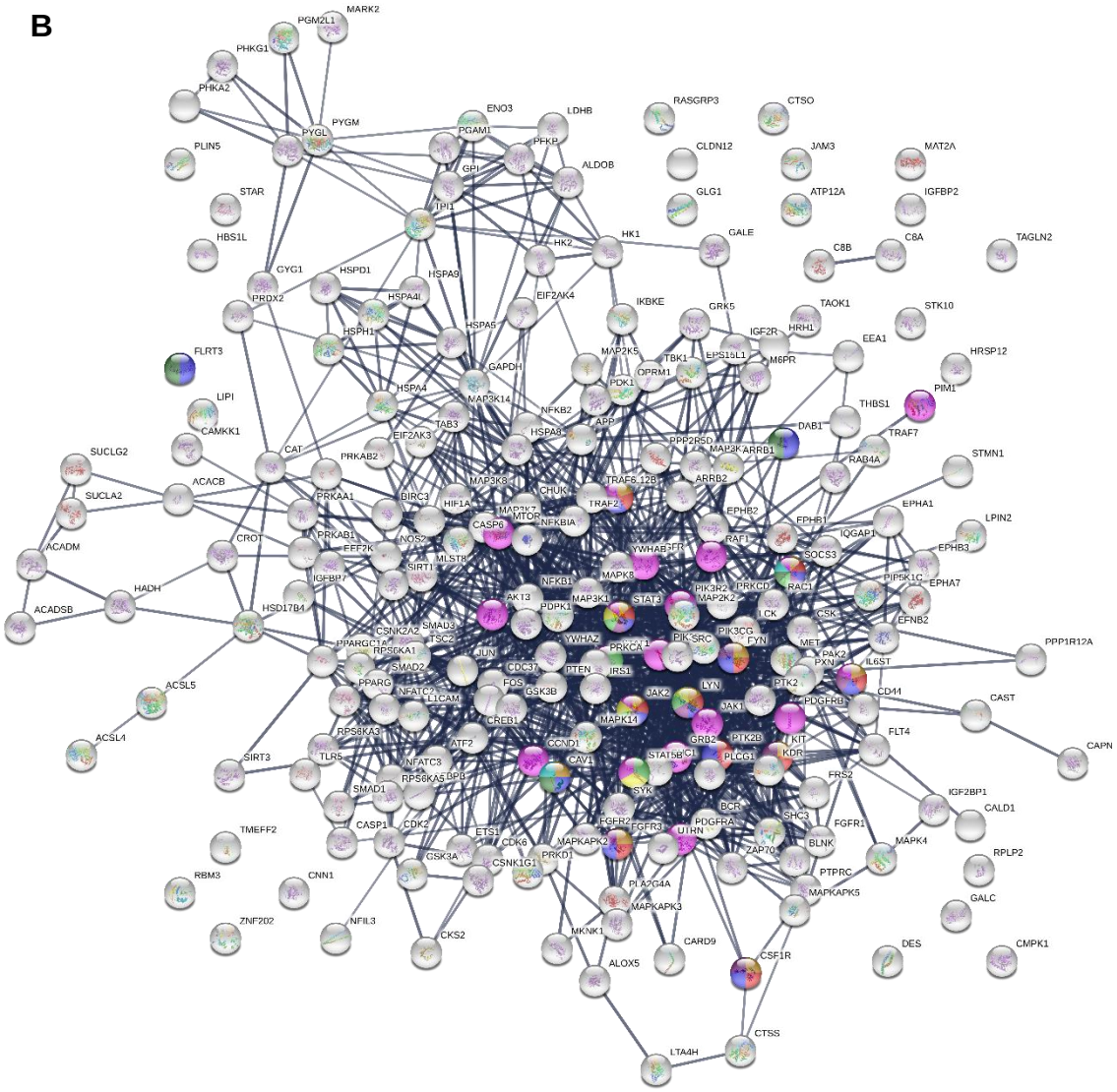

Figure 8 (continued).

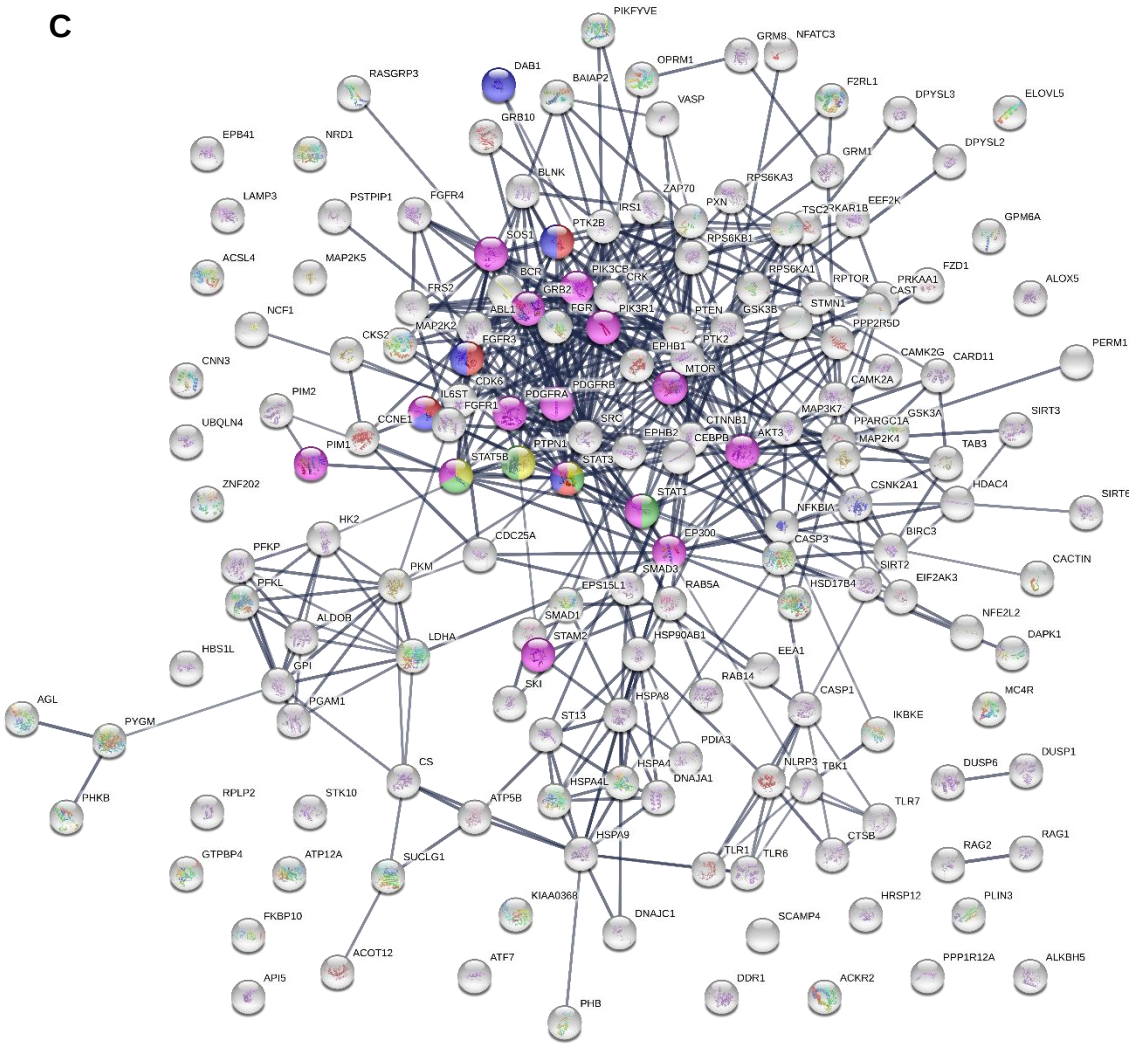

Figure 8 (continued).

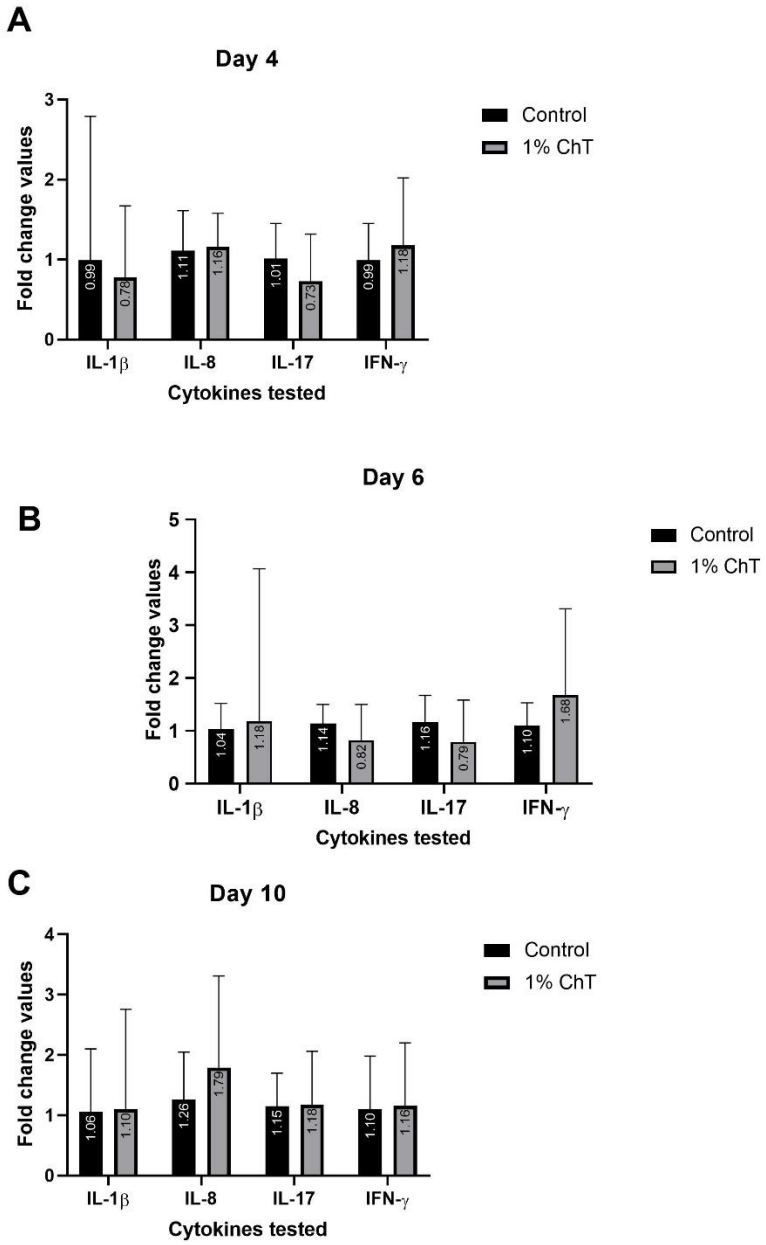

Figure 9. mRNA expression of cecal mRNA from experimental chicks fed 1% ChT inclusion diet, determined by quantitative RT-PCR.

(A) D4 fold changes by cytokines tested comparing averaged 1% ChT inclusion group with control group; (B) D6 fold changes by cytokines tested comparing averaged 1% ChT inclusion group with control group; (C) D10 fold changes by cytokines tested comparing averaged 1% ChT inclusion group with control group. Data represent the median values from two separate experiments. * = significantly different from the controls ($p \leq 0.05$).

The JAK family proteins are the initial receptors present to initiate the JAK-STAT pathway. In the chicken, JAK1 expression is found in the bursa and thymus at high levels perhaps due to the post-translational modification activity of JAK1 kinase in these locations (157). A previous study discovered interactions between avian JAK1 and STAT1/3/5B affected different cytokine families, including IL-6 and IL-10, in the gut during a disease state in the birds (158). Another study in broiler chickens found upregulated JAK1 and STAT1,3,5B mRNA levels led to increased expressions of IL-2, IL-4, IL-6, IL-7, IL-10, IL-22, and IFN-α (159). The mRNA expression data revealed an upregulation of statistically significant fold change on days 6 and 10 while the other proinflammatory cytokines were not found to be statistically significant. This provides evidence of an IL-6 immune mediated proinflammatory response, as opposed to initiating T cell receptors or chemokine receptors. Although **Table 10** shows that the T cell receptor signaling pathway and chemokine signaling pathway are the top two pathways with most altered peptides, this information only provides altered peptide numbers and not detailed phosphorylation or dephosphorylation events. The chemokine signaling pathway appeared to follow the CCR2-GNB network of signaling pathways; however, with the significantly increased phosphorylation in STAT1,3,5B peptides and significant phosphorylation presence of JAK2, this points to even greater evidence of the JAK-STAT pathway being turned on from the chemokine signaling pathway. As reflected by our mRNA expression results, chemokine CXCL-8/IL-8 did not show any significant fold change between the 1% ChT group and the control group. The T cell

receptor pathway showed less clear pathway information, even though it was the immune pathway with the most altered peptides. However, this pathway is an important precursor pathway that promotes signaling cascades for other immune pathways, including PI3K-Akt signaling pathway, MAPK signaling pathway, and NF-κβ signaling pathway (160). As shown in **Table 8**, the direct peptides involved in the pathway do not indicate a clear T cell function, which may be the reason why our IL-1 family cytokines did not show significant fold changes.

The kinome data provides further evidence in this trend with the dephosphorylation events within the T cell receptor signaling pathway and chemokine signaling pathway across all three days tested (**Table 9 and 10**). The kinomic data for day 6 seem to follow the IL-6 family of cytokine signaling for the JAK-STAT pathway: IL-6, which promotes growth and differentiation of different cell types, can activate the pathway by phosphorylating JAK1,2 and to then activate downstream signaling STAT family proteins, such as STAT1 and STAT3 (97,161). Interestingly, JAK-STAT signaling pathway was the only pathway that had IL6R out of the 13 signal pathways involved in avian embryonic stem cell differentiation (97). A previous *in vivo* experiment suggests that IL-6 can regulate the JAK-STAT pathway by decreasing JAK2 and increasing levels of STAT3(159,161), which is supported by our results. Our *in vivo* kinomic results (**Table 2**) observed phosphorylation of IL6ST on days 4 and 10, apart from dephosphorylation observed on day 6. This dephosphorylation may be due to the host immune system attempting to regulate the strong pro-inflammatory response via the IL-6 mediated pathway. Notably, SOCS3, the primary inhibitor of IL-6 signaling, is

present on days 4 and 6 in our data. The negative values as seen in this present study associated with the SOCS protein would indicate less presence of negative regulation of the JAK-STAT pathway (138,162,163). This would potentially explain the negative values associated with the JAK2 and IL6ST on days 4 and 6, respectively.

This study aimed to provide phosphorylation details regarding the broiler chick immune response when ChTs are included into the grower diet. The ChTs appear to be acting as immunomodulators due to the high upregulation of IL-6 proinflammatory cytokine. For practical application purposes, recent studies have suggested the usage of encouraging proinflammatory states for beneficial purposes of stimulating the immune system at early stages to improve resistance against pathogens (156). As a key signaling pathway involved in regulating innate immunity and adaptive immunity, the JAK-STAT pathway is crucial to study when considering ATAs, such as ChTs, for usage. Future experiments will investigate the usage of ChTs as antimicrobial immunomodulators on the local cecal level.

CHAPTER 6: CONCLUSIONS

As *Salmonella* Enteritidis continues to endure in poultry production, the necessity for identifying the host-pathogen interaction is crucial for pathogen control. The field of immunometabolism is gaining popularity but it is relatively new to date. The work presented herein was able to provide beneficial elucidations into the immunometabolic mechanisms of current host modulators utilized in industry: AGPs and ATAs. The research in this dissertation has substantially contributed to the field of immunometabolism in avian species by 1) identifying phenotypic change of the gut during *Salmonella* Enteritidis infection with a commonly used AGP in the current market, 2) providing proof of metabolic outcome when ChTs are introduced into the diet during early growth periods, and 3) elucidating the immune response by reviewing phosphorylation events of including ChTs into the diet.

While AGPs are banned in many countries, the mechanism of how they effectively promote growth and health is lacking in the literature for avian livestock species. Therefore, it is crucial to understand the mechanism of how it affects the immunometabolic host response before even thinking about ATAs. The current literature review outlines the wealth of information about *S. enterica* pathogenesis in mammalian species but there is a lack of detailed information in the chicken. Furthermore, the field of immunometabolism is relatively new, even for mammalian species. Thus, to further contribute to the field, the objective was to define the mechanisms of how *S.* Enteritidis affects immunometabolic tissue phenotype change in broiler chickens post-hatch.

As demonstrated by the first experiment, there is a definitive phenotype change in the ceca due to the shifting microbiota from including an AGP, specifically BMD, during *S.* Enteritidis infection. The mRNA expression data, providing genotype evidence, for BMD fed *Salmonella* challenged group (T4) demonstrated persistent pro-inflammatory response, unlike the classic phenotypic reprogramming at day 4 from pro-inflammatory to anti-inflammatory response. This immunological reprogramming is supported by the microbiota analysis: the decrease in *Enterobacteriaceae* and *Clostridiaceae* and increase of *Lachnospiraceae* indicate phenotypic change. This study demonstrated a unique phenomenon that has not been reported in the literature before, thereby contributing substantially to the field of immunometabolism.

However, due to increased incidence of antibacterial resistance, the efficacy of AGPs has been questionable. Therefore, a popular ATA candidate, ChTs, as a potential AGP replacement in the current livestock production was evaluated for its role in immunometabolic phenotype changes. In the fourth chapter, the results indicated an upregulation of IL-6, which is an important marker for not just immune pathways but also for metabolic pathways, demonstrating the important role that ChTs participate in host modulation via metabolism and immunity. To date, these are the first data in the literature demonstrating initial pathway data that supports growth and health promotion with ChT.

In the final chapter of this dissertation, further analysis was performed on the previous chapter to provide immune phosphorylation data of how ChTs affect the host. Through the utilization of KEGG and GO analysis, the results shown provided

meaningful proof of immunomodulatory activity of ChTs. Identifying the statistically significant immune phosphorylation events revealed the specific pathways that ChTs modulate in the host cecal tissue, namely the JAK-STAT pathway. This signaling pathway has been known to play an important role in growth factors, development, and cytokine activation (158). The results can provide useful information for utilizing ChTs as a potential ATA by its ability to target a crucial immune pathway.

The current work presented has also provided further advancement of an ATA in the current market, ChTs, by providing mechanistic data on how it modulates the host immunity. This advancement can potentially guide poultry production in providing safer, healthier meat products around the globe. However, further work needs to be performed to continue understanding the field of host-pathogen interaction. Future studies should look at the chicks' immunity from hatch to market and to test other segments of the GI tract for a wider overview of bacterial communities involved in immunometabolism. Furthermore, other immune and metabolic pathways should be analyzed for their role in host modulation. Our group is currently delving into scientific collaborations with academic and industry researchers in the poultry field. These future experiments will investigate the immunometabolic pathway involvement of other potential ATAs, food safety improvements, and mechanisms to develop downstream host-directed therapies. Overall, the field of immunometabolism needs to be further studied for any downstream application for human or livestock practical use.

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