APPLICATION OF TANNIC ACID AS A FEED SUPPLEMENT TO IMPROVE GUT HEALTH OF BROILER CHICKENS

by

JANGHAN CHOI

(Under the Direction of WOO KYUN KIM)

ABSTRACT

In broiler production, there are many challenging conditions (e.g., bacterial and parasitic infection) that can be detrimental to growth performance and gut health of broilers. Following the ban on the use of antimicrobial growth promotors (AGP) in 2006 in EU, it became essential to find alternative growth promotors with antimicrobial properties in broiler production. Tannic acid (TA), the standard of hydrolysable tannins, is considered a promising oral AGP alternative in broiler production due to its strong antimicrobial, antioxidant, anti-inflammatory effects. However, depending on the concentration used, TA can show favorable or detrimental effects in broilers because over-TA supplementation can reduce nutrient digestibility and feed intake and compromise gut health via precipitating dietary and endogenous nutrients. Therefore, the purpose of the study was to investigate effects of TA supplementation on growth performance, gut health, gut microbiota, bone health, body composition, and meat quantity and quality in broilers under healthy and challenging conditions. TA showed strong antimicrobial effects against *Salmonella* Typhimurium, and TA reduced swimming and swarming motility and biofilm formation against *Salmonella* Typhimurium in *in vitro* condition ($P < 0.05$). Approximately 10% of TA was recovered in the ceca of broilers. TA supplementation at concentrations greater than 972 mg/kg of

pelleted feed tended to reduce body weight in broilers D 21 ($P = 0.05$). TA supplementation at 1 to 2 g/kg of pelleted feed significantly reduced *S.* Typhimurium load in the ceca on D 7 compared to a challenged control group ($P < 0.05$), and average daily gain (ADG) tended to be linearly increased due to TA supplementation in broilers infected with *S.* Typhimurium ($P = 0.097$). TA supplementation linearly enhanced percentage of peripheral blood CD8+ T cells on D 18 ($P < 0.01$). TA supplementation at 0.5 and 2.75 g/kg significantly reduced oocyst shedding of *E. maxima* compared to the *E. maxima* infected control group ($P < 0.05$), and TA supplementation at 2.75 g/kg attenuated damaged gut permeability in broilers infected with *E. maxima* ($P < 0.01$). However, TA supplementation up to 2 g/kg in the pelleted feed linearly reduced BW in the starter and grower phase ($P < 0.05$) and increased fat deposition in broilers on D 42 ($P < 0.05$). Whereas TA supplementation has potential to improve growth performance and gut health in broilers under bacterial and parasitic infection condition, additional studies are needed in order to justify applying dietary TA in the field condition of broiler production to improve production efficiency.

Keywords: Chickens, broilers, tannic acid, antibiotic growth promotor alternative, gut health, and gut microbiota

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1.0. CHAPTER 1 GENERAL INTRODUCTION

Poultry meat is one of the most inexpensive and popular protein sources in the world (Wahyono and Utami, 2018). Consumption of poultry meat per capita in the world and in the US is approximately 14.7 kg (OECD-FAO) and 41.8 kg (USDA), respectively, which was higher than any other meat sources including pork, beef, sheep, and goat in 2019. Moreover, it is expected that worldwide poultry production would continue to increase annually (Naseem and King, 2018). With this boost in poultry meat production, there has been a significant enhancement in growth performance and feed efficiency of broiler chickens (Surai, 2020). However, poultry production continues to face diverse challenges including cost and environmental issues, bacterial and parasitic infection, heat stress, mycotoxins, etc., which can decrease production efficiency, resulting in a high carbon footprint, threat to public health, and a negative impact on animal welfare.

Currently, cost and sustainability are the leading important issues in the broiler industry. Whereas broiler meat is considered as the most cost-effective and sustainable animal protein sources due to efficient converting rate of feed to meat of chickens (Méda et al., 2021), the broiler production certainly requires more resources (e.g., feed, water, and land) and yet, still has environmental issues (e.g., manure, greenhouse gases, and ammonia) compared to the plant based and lab-grown meat, which are expected to be more popular in the near future (Van Loo et al., 2020). Moreover, the size of the broiler production is the larger than any other farm animals and is intense and growting annually, which would lead to more environmental issues worldwide. To compete with plant based and lab-grown meat in the future, the poultry production system needs to be cost-effective and sustainable with a shrinking carbon footprint. It would be ideal to achieve maximum output (favorable quantity and quality of meat) with minimum input (cheaper ingredients and shorter raising) by finding appropriate strategies to optimize nutrient utilization,

to attenuate negative effects of challenging conditions (bacterial and parasitic infection, heat stress, mycotoxins, etc.), and to mitigate environmental issues in broiler production.

Bacterial and parasitic infection via oral-fecal route is common in poultry production because chickens can be raised in non-hygienic conditions (e.g., reused litter and contaminated water) (El-Hack et al., 2021; Kyakuwaire et al., 2019). Bacterial and parasitic infections in poultry production remains as one of the most important issues to be resolved because they can reduce production efficiency and threat a public health via foodborne pathogens (Wigley, 2013). *Salmonella enterica* is one of the most predominant bacterial pathogens in poultry meat production (Faúndez et al., 2004). *Salmonella enterica* can contaminate chicken meat via invasion from the gastrointestinal tract, as well as contaminate meat product during the slaughter process (Humphrey et al., 1996; Rivera-Pérez et al., 2014). Moreover, a Salmonella infection can stimulate the immune system and disrupt gut eco-system of broiler chickens, which can result in reduced growth rate and feed efficiency of broilers (Vandeplas et al., 2010).

Coccidiosis is one of the predominant poultry diseases and induces severe worldwide economic losses estimated US $ 3 billion per year (Cunha et al., 2020). Coccidiosis is an enteric disease induced by Eimeria, a genus of apicomplexan parasite (Shirley et al., 2005). There are seven known Eimeria species causing hemorrhagic coccidiosis (*Eimeria tenella*, *Eimeria brunetti*, and *Eimeria necatrix)* and malabsorptive coccidiosis (*Eimeria acervulina*, *Eimeria maxima*, *Eimeria mitis*, and *Eimeria praecox*) (Blake and Tomley, 2014). Eimeria life cycle has two parts including endogenous and exogenous phase (Allen and Fetterer, 2002). After asexual and sexual reproduction of cycle of Eimeria in the endogenous phase, unsporulated oocysts are excreted via feces (Chapman, 2014). Unsporulated oocysts can be sporulated at appropriate temperature (30°C) and humidity of litter, and sporulated oocysts can re-infect chickens (Waldenstedt et al., 2001).

Infection of *Eimeria* spp. is known to severely decrease growth rate and damage gut health in broiler chickens (Teng et al., 2020b).

Diverse prevention and treatment strategies including vaccination, supplementing feed additives, and managing environmental factors (e.g., litter and water quality) are available to manage bacterial and parasitic infections in broiler production (Frederick and Huda, 2011; Peek and Landman, 2011). In the past, antibiotic growth promotors (AGP) and ionophore anticoccidials were commonly used to control bacterial and coccidial infections and to promote growth rate and gut health in broiler chickens (Robinson et al., 2019). However, due to the public concern on the spread of resistant pathogens and their genes to livestock animals and human populations, the use of AGP and ionophore anticoccidials was banned or restricted in many countries (Awaad et al., 2014). In the US and worldwide, the poultry industry is trying to establish an antibiotic-free production system due to the increase demands for antibiotic-free broiler meat product (Musa et al., 2020). However, there is no single bullet to completely replace AGP in broiler production at this point in time. In 2017, the worldwide sales of antibiotics in farm animals was estimated at 93,309 tons, with the sales expected to increase by 2030 (Tiseo et al., 2020). Moreover, sustainability of antibiotic free poultry production is in question because antibiotic free poultry production proper without coping strategies can result in reduced poultry production efficiency and an increase in food-borne pathogens, which can increase cost and environment issues and a threat to public health, respectively (Cervantes, 2015; Haque et al., 2020). Thus, it has been essential to find appropriate bioactive compounds to substitute AGP and anticoccidial ionophores in poultry production. These AGP alternatives should be eco-friendly and cost-effective and be able to promote growth rate and gut health of broilers under both challenging and non-challenging conditions (Yang et al., 2015b). Diverse bioactive compounds including amino acids (Teng et al.,

2021b), essential oils (Yang et al., 2021), organic acids (Kim et al., 2015), plant extracts (Mogire et al., 2021), prebiotics (Shang et al., 2015a), probiotics (Adhikari and Kim, 2017), and exogenous enzymes (Lu et al., 2020a) have been evaluated as AGP alternatives, and some of them are currently being used in poultry production. Presently, there are no bioactive compounds that can completely replace AGP and anticoccidial ionophores in poultry production. Having more antimicrobial options for broiler production may reduce the possibility of emergence of antimicrobial-resistant pathogens because pathogens are hard to develop resistant system against diverse antimicrobials (Ju et al., 2022). Moreover, blends of diverse antimicrobials are expected to exhibit synergistic effects because different mode of actions to exhibit antimicrobial effects and to promote growth performance of broilers can induce synergistic effects in animals (Choi et al., 2020c). Therefore, new studies are required to find bioactive compounds that can be used as AGP alternatives.

Tannins, polyphenol compounds found in plants that can precipitate proteins, can be generally categorized into hydrolysable and condensed tannins. Hydrolysable tannins include tannic acid (TA) and can be hydrolyzed in low and high pH as their name refers (Lotfi, 2020). However, condensed tannins are polymers formed from condensation of flavan and are not readily hydrolysable (Hagerman et al., 1992). Tannins are considered as promising AGP alternatives due to their strong antimicrobial, antioxidant, and anti-inflammatory effects (Redondo et al., 2014a). Traditionally, tannins were considered as anti-nutritional factors for broiler chickens because tannins can decrease feed intake and nutrient utilization mainly via inducing astringent taste and form complexes with diverse nutrients (e.g., proteins, minerals, and polysaccharides) (Huang et al., 2018). However, select tannin concentrations are expected to show beneficial effects on growth performance and gut health of animals (Huang et al., 2018). Thus, additional studies are required

to investigate the effects of tannins on growth performance and gut health in broilers as well as determine the appropriate concentrations of tannins to include as a feed supplement in broilers during different challenge conditions. The following literature review discusses the challenges that the poultry production has confronted and potential mitigation strategies by using tannins in broiler production.

2.0. CHAPTER 2 LITERATURE REVIEW

DIETARY APPLICATION OF TANNINS AS A POTENTIAL MITIGATION STRATEGY FOR CURRENT CHALLENGES IN POULTRY PRODUCTION[1]

[1] **Choi, J.**, and W. K. Kim. 2020. Dietary application of tannins as a potential mitigation strategy for current challenges in poultry production: A review. Animals 10:2389. Reprinted here with permission of the publisher.

2.1. ABSTRACT

The poultry industry has an important role in producing sources of protein for the world, and the size of global poultry production continues to increase annually. However, the poultry industry is confronting diverse challenges including bacterial infection (salmonellosis), coccidiosis, oxidative stress, including that caused by heat stress, welfare issues, such as food pad dermatitis (FPD), nitrogen and greenhouse gasses emissions that cumulatively cause food safety issues, all of which reduces the efficacy of poultry production, impair animal welfare, and generate environmental issues. Furthermore, the recent restrictions on the use of AGP have exacerbated several of these negative effects. Tannins, polyphenolic compounds that possess a protein precipitation capacity, have been considered as anti-nutritional factors in the past as high concentrations of tannins can decrease feed intake and negatively affect nutrient digestibility and absorption. However, tannins have been shown to have antimicrobial, antioxidant, and anti-inflammatory properties, and as such, have gained interest as promising bioactive compounds to help alleviate the challenges of AGP removal in the poultry industry. In addition, the beneficial effects of tannins can be enhanced by several strategies including heat processing, combining tannins with other bioactive compounds, and encapsulation. As a result, TA supplementation alone or in conjunction with the above strategies could be an effective strategy to decrease the need of AGP and otherwise improve poultry production efficiency.

2.2. INTRODUCTION

Poultry products including meat and eggs account for a significant part of global food production and constitute a protein staple throughout the world (Mottet and Tempio, 2017). The United States Department of Agriculture (USDA) reported in 2016 that global egg production was approximately 70 million tons, and poultry meat production reached more than 100 million tons,

which accounted for more than one-third of global meat production (Pawar et al., 2016). Moreover, global poultry production continues to increase annually (Mottet and Tempio, 2017). However, there are many challenges in the poultry industry including: bacterial infection (salmonellosis); parasitic infection (coccidiosis); oxidative stress, including that caused by heat stress; welfare issues such as food pad dermatitis (FPD); and nitrogen and greenhouse gas emissions which can cause severe economic losses, threaten food safety and public health, impair animal welfare, and induce environmental pollution (Akil and Ahmad, 2019; Hunter et al., 2017; Nkukwana, 2018; Pawar et al., 2016).

Antibiotic growth promoters (AGP) have been used as supplements in chicken diets to improve growth performance and gut health, predominantly due to their antimicrobial effects and immunomodulatory functions in chickens (Hassan et al., 2018; Kumar et al., 2018). However, because of the increased public concern about the transmission of antibiotic-resistant bacteria from poultry products, the use of AGP in poultry production has been banned or restricted in many countries (Dall, 2019; Flynn, 2011; Salim, 2017). In addition, some producers in the U.S. poultry industry have opted to entirely remove the use of antibiotics and instead raise chickens using "no antibiotics ever (NAE)" or "raised without antibiotics (RWA)" approaches (Feeks, 2019; Singer et al., 2019). As a consequence, the efficiency of poultry production has decreased due to an increase in various bacterial and parasitic infections and reductions in the growth rate of chickens (Cheng et al., 2014). In addition, because there is no "magic bullet" that can replace AGP, some poultry producers are still using antibiotics in the U.S. and in many other countries, and the use of antibiotics for livestock animals in the world is expected to increase, possibly owing to population growth which is associated with a greater demand for livestock products in middle-income countries (Van Boeckel et al., 2015). Therefore, it is essential to find alternatives to AGP, which

must be cost-effective, eco-friendly and have antimicrobial and growth-promoting effects, without causing side effects (e.g., generation of resistant bacteria) to the animals and humans (Yang et al., 2015a). Tannins, defined as polyphenolic compounds that can precipitate proteins, are secondary metabolites, which are found in plants, seeds, bark, wood leaves and fruit skins and serve as plant defense mechanisms against predation (Redondo et al., 2014b). High concentrations of tannins have been shown to have anti-nutritional effects in monogastric animals because tannins can decrease feed intake, nutrient digestibility, and growth performance of chickens (Garcia et al., 2004; Trevino et al., 1992). However, recently in poultry production, tannins have garnered a great deal of attention as an alternative for AGP because of their antimicrobial, antioxidants, and anti-inflammation properties (Amirmohammadi et al., 2014; Brus et al., 2018; Daglia, 2012). In addition, many tannins are considered sustainable feed additives, as they are derived from byproducts of plant-based agriculture and industry. For example, chestnut tannins, which are already a supplement in poultry, are obtained by the distillation of wood that is used in the building industry (Mannelli et al., 2019). However, the effects of tannins on the growth performance and gut health of the chickens are still inconsistent and their mode of action is unclear. Therefore, it is important to understand the chemical properties and biological effects of tannins to maximize the use of supplemental tannins in chickens. This review is mainly focused on the classification and bioavailability of tannins, the effects of distinct tannins on mitigating the challenges facing poultry production, and strategies to enhance the effects of tannins.

2.3. CLASSIFICATION AND BIOAVAILABILITY OF TANNINS

Tannins, defined as polyphenolic compounds that have a protein precipitation capacity, exist in several different types with various molecular weights (Haslam, 1989). Plant tannins are classified into hydrolysable tannins (HT) with tannin derivatives (e.g., gallic acid and ellagic acid)

and condensed tannins (CT) (Huang et al., 2018) (Figure 2.1.). Additionally, phlorotannins (PT) are a third class of tannins unique to brown algae. Tannins have different bioavailability (absorbability), and their level of bioavailability varies depending on several factors, including the derivatives of each tannin (e.g., gallic acid and ellagic acid), their affinity to protein, molecular structure, and molecular weight. The bioavailability of tannins is an important trait for their functionality and should be considered for tackling different issues in poultry production. Tannins with a low bioavailability potentially have better antimicrobial effects in chickens, whereas highly bioavailable tannins would be more beneficial as antioxidant and anti-inflammatory agents.

The HT including predominately gallotannins and ellagitannins (molecular weight 500 to 3,000 Da) contain a polyol (normally glucose) as a central core, which is esterified with phenolic groups (e.g., gallic acid and ellagic acid) (Patra and Saxena, 2011). Under certain conditions (e.g., thermal processing, esterification, and acid or base treatment), HT can be hydrolyzed to yield free gallic acid and ellagic acid. Gallotannins produce one molecule of sugar and 9 to 10 molecules of gallic acid when hydrolyzed, whereas ellagitannins yield one molecule of sugar and several molecules of gallic acid and ellagic acid (Lamy et al., 2016). The unhydrolyzed HT are partially absorbable in the small intestine (Kamiloglu et al., 2016). Gallic acid is readily absorbable into the blood stream (Manach et al., 2005), while ellagic acid has low bioavailability due to strong affinity for proteins and poor absorption (Seeram et al., 2004). Hence, HT include gallotannins and ellagitannins and have different bioavailability dependingbased on the components and structure.

The CT are defined as oligomeric or polymeric flavonoids containing flavan-3-olunits such as catechin, epicatechin, gallocatechin, and epigallocatechin (Huang et al., 2018). The CT exist in fruits (e.g., berries, pears, and apples), forage legumes (e.g., lentils, black-eyed peas, chickpeas and red kidney beans), nuts, red and green grapes (and their juice and wine) (Costain et al., 2001;

Martinez et al., 2017; Selma et al., 2009). The CT have higher molecular weight (1,000—20,000 Da) and more complex structure compared to HT (Huang et al., 2018). Unlike HT, CT are not susceptible to hydrolyzation, which may imply low bioavailability in the gastrointestinal tract (GIT) of the chickens. This is because tannins of high molecular weight are hardly absorbable in the intestine (Serrano et al., 2009). Kahle et al. (2007) reported that around 90% procyanidins (CT) were recovered in the distal ileum, which imply that most of CT can reach to the large intestine. CT in the large intestine can be metabolized and absorbed by epithelial cells (Bawadi et al., 2005). CT have low bioavailability compared to HT and can be delivered to the large intestine of the chicken.

PT, algae-derived polyphenols, have molecular sizes ranging 126 Da to 650 kDa, but most of them are 10 and 100 kDa (Kirke et al., 2017; Ragan and Glombitza, 1986). Using *in vitro* models, Corona et al. (2017) demonstrated that PT can be metabolized and absorbed in the upper GIT potentially due to its low molecular weight, and limited amounts of biologically active PT were delivered to the colon. In addition, the author also demonstrated that higher molecular weight of PT are less susceptible to metabolization and absorption in the upper GIT (Corona et al., 2017). Nwosu et al. (2011) showed that PT can be metabolized and absorbed by the colon cells (e.g., Caco-2). Therefore, PT can be easily metabolized and absorbed in the GIT tracts due to their low molecular weights. However, future *in vivo* experiments are required to study bioavailability of PT in chickens.

Taken together, tannins are classified into HT, CT and PT, and their bioavailability depending on their components, structure, and molecular weight. It is important to understand specific chemical properties each tannin prior to application in poultry diets.

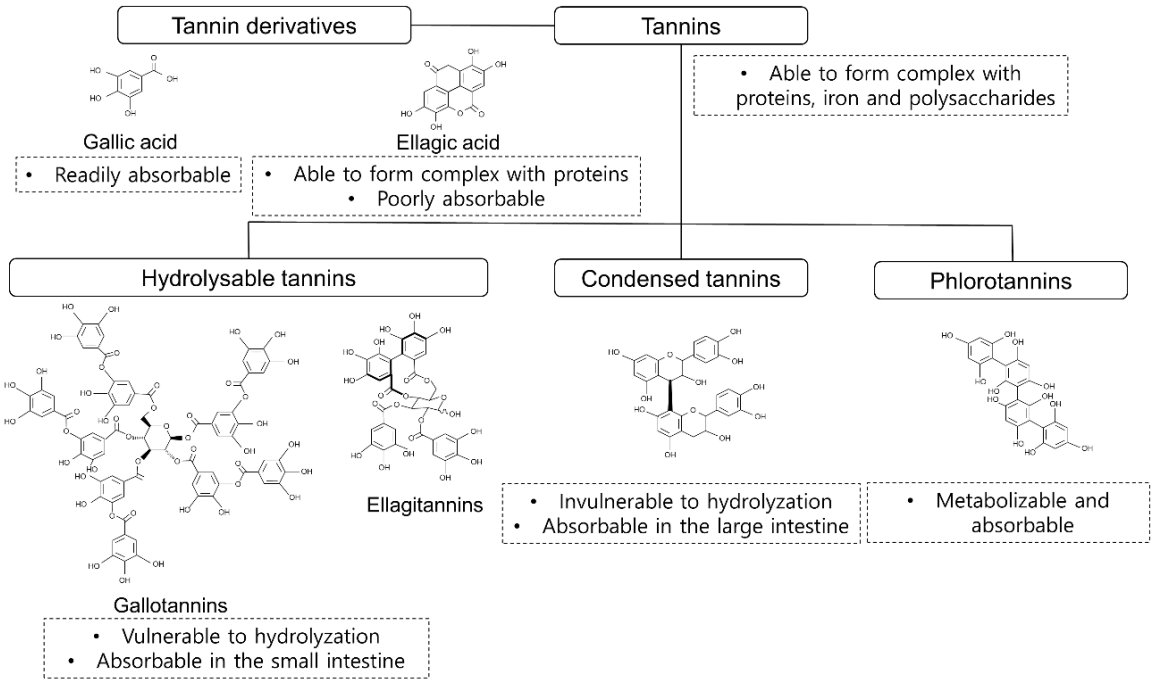


Figure 2.1. Classification and bioavailability of tannins. Hydrolysable tannins and condensed tannins belong to plant tannins, and phlorotannins are found in brown algae.

2.4. TRADITIONAL POINT VIEW OF TANNINS AS ANTI-NUTRITIONAL FACTORS IN POULTRY PRODUCTION

While tannins are recognized as beneficial bioactive compounds in ruminants, they have been considered to have anti-nutritional properties in poultry diets (Redondo et al., 2014b). In the ruminant GI tract, tannins bind proteins under rumen pH (pH 5.5–7.0), inhibiting the microbial degradation of dietary proteins. Upon reaching the abomasum (pH 2.5–3.5), the non-covalent linkages between proteins and tannins are broken, and free protein can be absorbed by the host in the distal small intestine (pH 7.5) (Barry et al., 1986). As such, tannins are known to increase ruminant's protein utilization and decrease gas emission (Berard et al., 2011; Goel and Makkar, 2012). However, in chickens, tannins hve been classified as phytotoxins predominately due to their protein binding properties, which impairs dietary protein digestion and decreases digestive enzyme activity (Medugu et al., 2012). Moreover, tannins bind proline-rich, hydrophobic salivary proteins of chickens, forming complexes that are responsible for an astringent taste of the feed, and in turn lead to low palatability and decreased feed intake of chickens (Butler et al., 1984). In addition, a high concentration of tannic acid (25 g/kg) showed toxicity to chickens by altering liver proteolytic activity in broiler chickens (Marzo et al., 2002). Lee et al. (2010) reported that tannic acid impaired growth performance, hematological indices, and plasma iron status in weanling pigs since tannic acid can form complex with iron. However, these toxic properties were reported in tannin diets greater than 7.5 g/kg (based on tannic acid, considered as the standard of HT). Still a number of studies proved that appropriate amounts of tannins (based on tannic acid; standard of tannins) ranging from to 0.5 g/kg to 5 g/kg in poultry could improve growth rate and gut health due to their potential antimicrobial, antioxidants and anti-inflammatory functions (Table 2.1.). Therefore, while in the past, tannins

were considered to have anti-nutritional properties, tannins at appropriate concentrations have the potential to improve growth performance and gut health of chickens.

Table 2.1. Effects of different concentration of tannic acid on the broiler chickens

Concentrations of tannic acid	Outcomes	References
0.5 g/kg	Improved growth performance and immune system in broiler chickens challenged with *Eimeria* spp.	(Tonda et al., 2018)
2 g/kg	Improved foot pad dermatitis of the chickens without affecting growth performance	(Cengiz et al., 2017)
5 g/kg	Improved growth performance and decreased lipid oxidation.	(Starčević et al., 2015)
5 g/kg	Increased growth performance, modulated cecal microbial metabolites and decreased cecal pH.	(Mašek et al., 2014)
7.5 and 15 g/kg	Decreased growth performance and did not inhibit the growth of *Salmonella* Typhimurium in broiler chickens challenged with *S.* Typhimurium.	(Kubena et al., 2001)
10 g/kg	Attenuated fatty acid profile of breast but decreased growth performance in the heat stressed chickens.	(Ebrahim et al., 2015)
25 g/kg	Decreased growth performance and showed liver toxicity by inducing liver proteolytic activity	(Marzo et al., 2002)

2.5. CHALLENGES IN POULTRY PRODUCTION AND POTENTIAL SOLUTIONS BY USING TANNINS

2.5.1. Effects of tannins on bacterial infection (Salmonellosis)

There are many pathogenic bacteria in chickens and poultry products (meat and eggs), including *Salmonella* Typhimurium, *Escherichia coli* O157:H7, *Campylobacter jejuni*, *Clostridium perfringens*, enterohemorrhagic *Escherichia. coli* (EHEC), *Listeria monocytogenes*, *Arcobacter butzleri*, *Mycobacterium avium* subsp. Paratuberculosis and *Aeromonas hydrophila* (Mor-Mur and Yuste, 2010). These pathogenic bacteria not only impair gut health and growth rate of chickens, but are also a public health threat as foodborne diseases in humans (Adhikari et al., 2018). Salmonellosis, one of the main food-borne diseases from poultry products, is induced by *S.* Typhimurium and *S.* Enteritis and causes, illness, morbidity and mortality in humans (Thung et al., 2016).

Numerous *in vitro* studies have shown that tannins and their derivatives showed bacteriostatic (inhibiting bacterial growth) and bactericidal (killing bacteria) effects on *Salmonella* spp. and other pathogens, as shown in Table 2.2. Potential mechanisms of antibacterial effects of tannins include (1) direct interactions with components in the cell wall to alter morphology of the cell wall and to increase membrane permeability of bacteria (Huang et al., 2018); (2) decreasing activities of microbial enzymes (Wu-Yuan et al., 1988); and (3) depriving essential nutrients such as proteins and minerals (e.g., iron) for pathogenic bacteria (Delimont et al., 2017; Karamać, 2009; Tan, 2019). In addition to bacteriostatic and bactericidal effects of tannins, many *in vitro* studies reported that sub-lethal concentrations of tannins also restricted pathogenicity of *Salmonella* spp. and other pathogens by inhibiting motility (Mahadwar et al., 2015), quorum sensing (Sivasankar et al., 2020) and biofilm

formation (Yang et al., 2016a) of pathogenic bacteria.

Table 2.2. *In vitro* antimicrobial effects tannins against diverse pathogenic bacteria

Tannins sources	Strains	Results and conclusions	References
Ellagitannins from Chestnut wood Gallotannins from Tara and Sumach Condensed tannins from Quebaracho, Tannino and *Calliandra calothyrsus* Flavanol gallates from Tea and *Acacia nilotica*	*Salmonella* Typhimurium	All of the tannins inhibited the growth of *S.* Typhimurium.	(Costabile et al., 2011)
Tannic acid Gallic acid	*S.* Typhimurium	Tannic acid and gallic acid had bactericidal effects and gallic acid had higher bactericidal effects than tannic acid	(Reyes et al., 2017)
Condensed tannins extracted from tree leaves viz. babool (*Acacia nilotica*), jamun (*Eugenia jambolana*), peepal (*Ficus religiosa*), subabul (*Leucaenia leucocephala*), and guajava (*Psidium guajava*)	*Escherichia coli* *Staphylococcus aureus* *S.* enteritis *Enterococcus faecalis*	All of the selected five condensed tannins inhibited the growth of the four pathogenic bacteria	(Daing et al., 2017)
Tannin extracts from *Cytinus hypocistis* and *C. ruber*	*S. aureus* *S. epidermidis* *E. faecium* *Pseudomonas aeruginosa* *Klebsiella pneumoniae*	Tannin extracts from *C. hypocistis* and *C. ruber* showed antibacterial and antibiofilm activities against gram positive and negative human pathogens.	(Maisetta et al., 2019)
Chestnut tannins (80% hydrolysable tannins) Quebracho tannins (75% condensed tannins)	*Clostridium perfringens*	Both hydrolysable and condensed tannins showed antimicrobial effects against C. perfringens and neutralized its cytotoxicity.	(Elizondo et al., 2010)

In addition to their antibacterial effects, tannins can control systemic infection of *Salmonella* spp. by beneficially altering select components of gut health. Salmonella spp. can enter blood circulation via paracellular and transcellular pathways and use immune cells to enter enterocytes to be distributed in the internal organs and muscles in chickens (Figure 2.2.). Many studies reported that tannins altered expression and functionality of tight junction proteins (Liu et al., 2018), mucus (Kamali Sangani et al., 2014; Moghaddam et al., 2011), and immune cells (Li et al., 2019b; Urdaneta and Casadesús, 2017) of chickens. Nevertheless, supplemental tannins have not typically translated to antimicrobial effects against Salmonella spp. in *in vivo* studies. For example, Van Parys et al. (2010) reported that HT extract of sweet chestnut woods exhibited strong antimicrobial effects (minimum inhibitory concentration: 25–50 μg/mL and minimum inhibitory concentration: 100 μg/mL) against *S.* Typhimurium isolated from pigs under *in vitro* conditions, the inclusion of 3,000 mg/kg HT in the pig feed did not reduce the *Salmonella* spp. concentration in feces, intestine and internal organs of pigs inoculated 10^7 colony forming units (CFU)/mL of *S.* Typhimurium strain at four days post-inoculum. Similarly, Kubena et al. (2001) reported that tannic acid (7.5 or 15 g/kg in the feed) did not modulate the salmonella concentration in cecal content of broiler chickens inoculated with 10^4 CFU of *S.* Typhimurium. Potential explanations for such findings include (1) tannins were degraded by host or microbial enzymes or absorbed before they reached the lower intestine where most of the *Salmonella* spp. and other pathogenic bacteria propagate; (2) tannins formed complexes with components of feedstuffs (polysaccharides and proteins) or endogenous proteins, which inhibits antimicrobial effects of tannins; and (3) experimental factors including the low concentrations of tannins, the high concentrations of salmonella inoculum or short period of the experiments were possibly obstacles to diminish the

antimicrobial effects of tannins in *in vivo* models (Van Parys et al., 2010). In contrast, Jamroz et al. (2009) reported that 1,000 mg/kg of sweet chestnut tannins reduced the number of E. coli and coliforms bacteria in the small intestine on 28 d; however, growth performance of the tannin-fed birds was decreased in this study. While many *in vitro* studies have reported that tannins have antimicrobial effects against *Salmonella* spp. and other pathogens, the optimal antimicrobial concentrations in chickens have not yet been fully determined. Therefore, dose-specific antimicrobial effects of tannins in chickens, as well as their mode of actions, warrant further investigation.

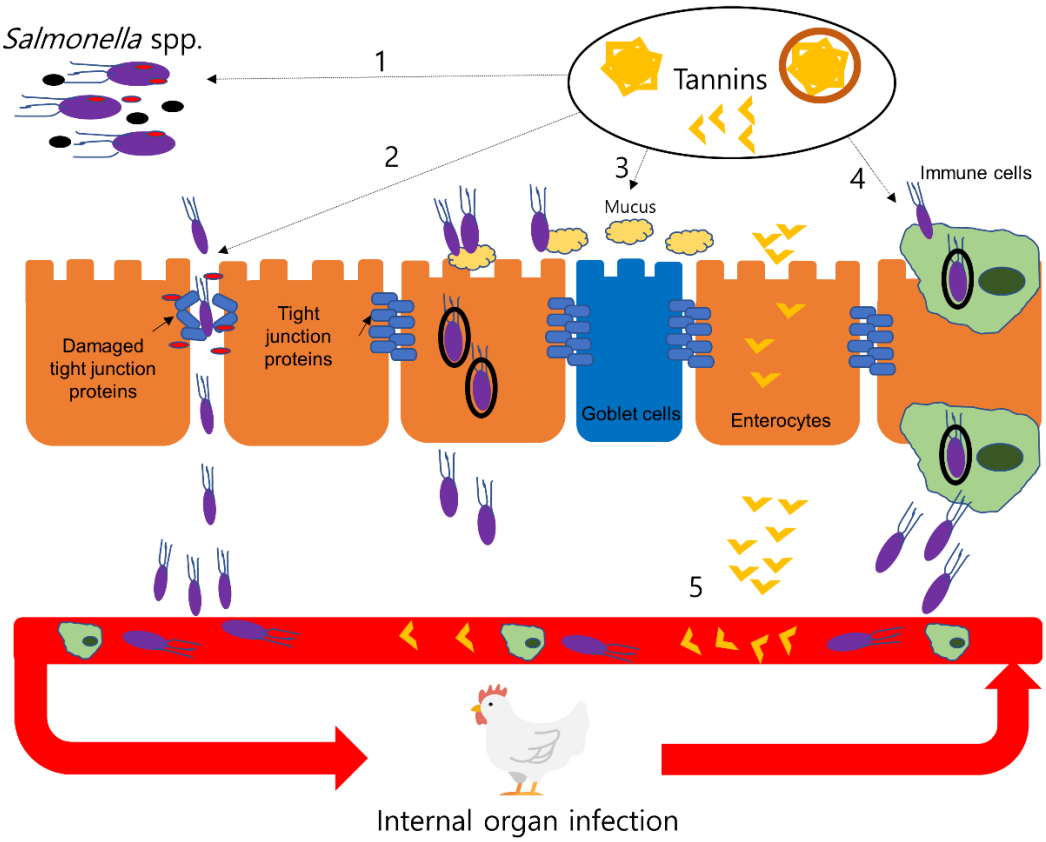


Figure 2.2. Systemic infection routes of *Salmonella* spp. and potential mechanisms of antibacterial actions of tannins in chickens. Chickens can orally ingest *Salmonella* spp. from feed or their environment, and subsequently escape the small or large intestine (Adhikari et al., 2017). (1) Tannins and tannin derivatives (tannins, hydrolyzed tannins, and tannin-protein complexes) are known to inhibit the growth of *Salmonella* spp. in the intestine and decrease quorum sensing of the bacteria (Sivasankar et al., 2020). Lipopolysaccharides (LPS) of *Salmonella* spp. can impair intestinal barrier function, which allows *Salmonella* spp. to pass the paracellular pathways of the intestine (Chen et al., 2018b; Omonijo et al., 2018b). (2) Tannins and tannin derivatives may improve gut barrier integrity by neutralizing LPS or decreasing expression of cytokines, which can impair tight junction proteins (van Ampting et al., 2010). (3) and (4) *Salmonella* spp. also utilized mucus and immune cells to invade epithelial

cells (Urdaneta and Casadesús, 2017). Tannins and their derivatives potentially modulate expression and morphology of immune cells and mucus. *Salmonella* spp. can invade epithelial cells in diverse pathways and enter blood circulation and finally colonize in internal organs (e.g., liver, kidney, spleen, etc.) and muscles in chickens (Adhikari et al., 2019). (5) Tannins also can enter the blood circulation and potentially show antimicrobial effects and modulate immune cells to attenuate internal organ infection by *Salmonella* spp. in chickens (Konishi et al., 2003; Sieniawska and Baj, 2017).

2.5.2. Effects of tannins on coccidiosis

Coccidiosis, which is a parasitic disease induced by protozoa of the family Eimeridae, is one of the most prevalent and detrimental enteric diseases in poultry production (Grilli et al., 2018). Nine known Eimeria species in chickens include *E. acervulina*, *E. brunetti*, *E. maxima*, *E. necatrix*, *E. praecox*, *E. mitis*, *E. tenella*, *E. mivati*, and *E. hagani* to date (Chapman, 2014; Clark et al., 2016). *Eimeria* spp. infect and multiply within the mucosal epithelial layers in different parts of the GIT through the fecal-oral route (Li et al., 2019a). Coccidiosis can result in reduced growth rate and gut barrier integrity and induce inflammation, diarrhea, hemorrhaging, and even mortality in broiler chickens, negatively influencing the efficacy of poultry production and welfare (Teng et al., 2020b; Yin et al., 2014). The negative effects of coccidiosis on gut health of chickens are closely related to oxidative stress, as Eimeria infections cause lipid peroxidation and excessive production of reactive oxygen species (ROS) in chickens (Abbas et al., 2013). Furthermore, coccidiosis is closely associated with an enteric infectious disease, necrotic enteritis, which is predominately induced by C. perfringens with the presence of *Eimeria* spp. (Prescott et al., 2016; Wu et al., 2014). To address coccidiosis for the poultry industry, prophylactic coccidiostats and anticoccidial drugs have been supplemented in poultry diets (Peek and Landman, 2011). However, resistance for all currently available drugs have been documented, making it imperative to discover novel drug alternatives that induce limited resistance and effectively control coccidiosis in broiler chickens (Noack et al., 2019). To find alternatives for prophylactic coccidiostats and anticoccidial drugs, bioactive compounds including prebiotics (Levine et al., 2018), plant extracts (Christaki et al., 2004), organic acids (Abbas et al., 2011), essential oils (Yang et al., 2020), lipids (fatty acids) (Bortoluzzi et al., 2018), minerals (e.g., zinc) (He et al., 2019), and

nitro compounds (Teng et al., 2020a) have been studied in chickens.

Tannins are known to have anticoccidial effects because tannins can form complexes with parasitic enzymes and metal ions, which are essential for *Eimeria* spp. and can stimulate the chicken immune system (Chung et al., 1998; Min and Hart, 2003; Scalbert, 1991). Tonda et al. (2018) reported that the dietary supplementation of 500 mg/kg of gallnut tannic acid extract reduced total oocyst number in excreta, and 500 mg/kg of tannic acid or gallnut tannic acid extract decreased intestinal lesion scores in broilers infected with *Eimeria* spp. Furthermore, the authors showed that gallnut tannic acid extract enhanced feed conversion ratio of cocci-vaccinated birds, which possibly implies that gallnut tannic acid extract improved protective immunity following coccidiosis vaccination (Tonda et al., 2018). Supplemental chestnut HT and quebracho CT tannins reduced Eimeria spp. oocyst shedding and parasitic bacterial diarrhea and attenuated negative effects of coccidiosis via immunomodulating and anti-inflammatory effects in rabbits (Parisi et al., 2018). In broiler chickens, Kaleem et al. (2014) showed that administration of *Emblica officinalis* derived tannins improved growth performance and showed immunostimulatory properties and enhanced immunity. The beneficial effects of tannins on gut health of chickens infected with *Eimeria* spp. are closely associated with antioxidant properties of tannins, which can restore an Eimeria-damaged gastrointestinal (Mishra and Jha, 2019). A study by Wang et al. (2008) demonstrated that supplementation of grape seed proanthocyanidin extract, rich in CT, enhanced growth performance and attenuated clinical symptoms, potentially by improving antioxidant capacity in chickens infected with *Eimeria tenella*. In contrast, Mansoori and Modirsanei (2012) showed that supplemental tannic acid (10 g/kg) numerically increased D-xylose absorption in chickens vaccinated against coccidiosis followed by challenging with *Eimeria* spp., however,

supplemental tannic acid increased the total number of oocysts in excreta, indicating that high concentrations of tannins can attenuate the efficiency of anticoccidial vaccines and impair an appropriate immune response against coccidiosis in chickens. This discrepancy may be attributed to different sources and concentrations of tannins and dissimilar experimental conditions (e.g., challenge concentrations of *Eimeria* spp.).

Although many studies reported the potential benefits of supplemental tannins in broiler chickens infected *Eimeria* spp., more comprehensive studies are required (1) to study the mechanisms under anticoccidial effects of tannins in chickens; (2) to elucidate mechanisms of the beneficial effects of tannins on the gut health of chickens infected with *Eimeria* spp.; (3) to investigate the effects of tannins in a necrotic enteritis challenge model by using inoculum of *Eimeria* spp. and *C. perfringens*; and (4) to find appropriate concentrations and types of tannins against coccidiosis in chickens.

2.5.3. Effects of tannins on heat stress and oxidative stress

Heat stress is one of the major obstacles in the poultry industry because it negatively impacts growth performance, gut health, meat quality, and welfare of chickens (Attia et al., 2017; Quinteiro-Filho et al., 2010). A potential reason for negative effects of heat stress on chickens is closely associated with excessive production of ROS via accelerated metabolic reactions due to mitochondrial respiration (Choi et al., 2020a; Laudicina and Marnett, 1990; Lin et al., 2006). Under normal conditions, enzymatic and non-enzymatic antioxidants can neutralize ROS and maintain an optimal balance between oxidants and antioxidants (Lauridsen, 2019; Lee et al., 2018) (Figure 2.3.). However, if there is an imbalance between oxidants and antioxidants in chickens, increased production of ROS can impair gut health and induce inflammation, which results in decreased growth performance of chickens (Nawab et al., 2018).

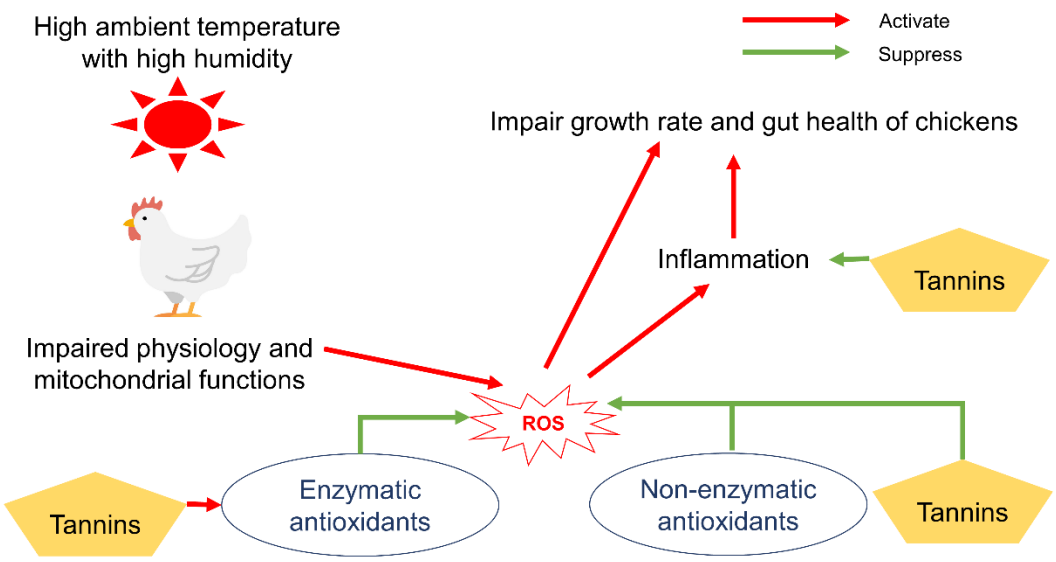


Figure 2.3. Potential mechanisms for the alleviating effects of tannins in heat-stressed chickens. High ambient temperature with high humidity increases core body temperature of chickens, and this can result in impaired physiology and mitochondrial functions, which lead to excessive reactive oxygen species (ROS) (Mujahid et al., 2005). Tannins, polyphenolic compounds, directly scavenge ROS like non-enzymatic antioxidants (Nakagawa and Yokozawa, 2002), and also, tannins can increase activities of antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPX), and glutathione reductase (GR) in broiler chickens. The ROS cause inflammation that can impair growth rate and gut health of chickens and tannins also have anti-inflammatory function (Sahin et al., 2010).

Antioxidants including plant extracts (Jiang et al., 2019; Yang et al., 2019), L-carnitine (Terruzzi et al., 2019), Vitamin C (Sahin et al., 2003; Yun et al., 2012), Vitamin E (Kumbhar et al., 2018), and selenium (Shakeri et al., 2019) have been studied to alleviate heat stress and oxidative stress.

Tannins are believed to relieve or attenuate effects of oxidative stress, including that caused by heat stress by scavenging ROS and modulating enzymatic antioxidants in animals (Figure 2.3.) (Yang et al., 2019). The reducing power of tannins including proanthocyanidins, catechins, epicatechin, and procyanidin from grade seeds is approximately 20 times higher than Vitamin E and 50 times higher than Vitamin C (Shi et al., 2003). A study from Sahin et al. (2010) reported that 200 or 400 mg/kg of epigallocatechin-3-gallate (the ester of epigallocatechin and gallic acid) from green tea decreased oxidative stress by controlling the hepatic nuclear transcription factors such as nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) and nuclear factor (erythroid-derived 2)-like 2 (Nrf2) in heat-stressed quail. Furthermore, 10 mg/kg of tannic acid improved fatty acid profile (decreased monosaturated fatty acids) in breast muscle of chickens under heat stress (Ebrahim et al., 2015). Ramnath and Rekha (2009) showed that supplementation of *Brahma Rasayana* containing various sorts of tannins enhanced activities of enzymatic antioxidants including superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR), and reduced glutathione (GSH) in the blood of chickens subjected to cold temperatures. Moreover, the inclusion of grape (Vitis vinifera) pomace, rich in CT, enhanced antioxidant enzyme activities (GPx and SOD) and intestinal morphology and increased relative weight of bursa of Fabricius and thymus in the heat-stressed broiler chickens (Hosseini-Vashan et al., 2020). In a diquat-induced mouse model (oxidative stress model), tannic acid improved intestinal morphology, activated the

antioxidative pathway by reducing protein expression of Kelch like-ECH-associated protein 1 (KEAP1) and enhancing protein expression of nuclear factor erythroid 2-related factor 2 (NRF2), as well as modulated intestinal barrier function in the jejunum (Wang et al., 2019). Thus, supplementing appropriate concentrations of tannins would potentially be an effective strategy to attenuate oxidative stress in heat-stressed birds.

2.5.4. Effects of tannins on food pad dermatitis

Foot pad dermatitis (FPD) is defined as a condition that induces lesions on the plantar surface of the footpads in growing chickens (Shepherd and Fairchild, 2010). FPD causes severe economic losses in poultry production because foot pads are the third most crucial economic component in broiler chickens, and FPD can impair growth rate, gut health, and welfare of chickens (Abd El-Wahab et al., 2013; Shepherd and Fairchild, 2010). Thøfner et al. (2019) showed that FPD and systemic bacterial infections are closely correlated because pathogens can gain entry into the chickens through damaged epithelium on the foot pads (Heidemann Olsen et al., 2018). Litter moisture and litter quality are two crucial factors that cause FPD as well as bedding materials and depth, drinkers, and nutrient deficiencies (El-Wahab et al., 2012; Mayne et al., 2007a; Shepherd and Fairchild, 2010). In addition, increased excreta viscosity has been known to increase the occurrence and severity of FPD in chickens by altering litter moisture and litter quality (Cengız et al., 2012).

Tannins can relieve the incidence and severity of FPD by enhancing fecal consistency (e.g., fecal dry matter contents) and litter quality (Redondo et al., 2014b). Cengiz et al. (2017) reported that 2,000 mg/kg of tannic acid reduced the incidence and severity of FPD in broiler chickens without affecting growth performance, litter quality and intestinal viscosity of chickens. Moreover, 700 mg/kg and 2,000 mg/kg of tannin-rich sweet chestnut wood extract

increased fecal dry matter contents in chickens (Rezar and Salobir, 2014). In addition, antimicrobial, antioxidant, and anti-inflammatory properties of tannins probably helped to attenuate the incidence and severity of FPD in broiler chickens because oxidative stress and inflammation can exacerbate the severity and consequences of FPD in chickens (Mayne et al., 2007b; Salmond, 2015). Thus, TA supplementation has the potential to reduce severity and incidence of FPD in broiler chickens by enhancing fecal consistency and litter quality.

2.5.5. Effects of tannins on nitrogen excretion and emissions of noxious and greenhouse gases

Reactive nitrogen species (ammonia, nitrous oxide, and other oxides of nitrogen) and sulfur containing compounds (hydrogen sulfide and sulfur dioxide) are produced in poultry production facilities and cause environment pollution and greenhouse gases such as carbon monoxide, carbon dioxide and methane (Bostami et al., 2015; McCrory and Hobbs, 2001; Wang and Huang, 2005; Wang et al., 2011). Factors affecting the production of nitrogen compounds and detrimental gases include types of feedstuffs, manure conditions, and housing accessories (bedding and heating materials) (Aneja et al., 2012; Xin et al., 2011). Nitrogen and greenhouse gases are produced in the livestock animals due to enteric fermentation and manure fermentation, and ruminants contribute most of the livestock greenhouse gas production because of high fermentation rate in the rumen (Wang and Huang, 2005). Although poultry production is not a major contributor of noxious or greenhouse gases, the continued growth of poultry industry coupled with higher intensity of excretion and emission per unit compared to other species, mandates the need to find strategies to mitigate the production of nitrogen excretion and emissions of noxious and greenhouse gases (Malomo et al., 2018).

It is well-established that tannins improve N utilization and decrease methane production in ruminant animals (Aboagye et al., 2019; Patra and Saxena, 2011). The potential

mechanisms underlying effects of tannins on mitigation of emissions of nitrogen and greenhouse gases are inhibiting growth of methanogens, reducing protozoal-associated methane production, and decreasing fiber fermentation (Patra and Saxena, 2011). Although chickens (monogastric animals) have a distinct GI tract from ruminants, tannins may alter gut health and microbiota and improve N utilization in chickens, thereby potentially reducing nitrogen and methane emissions. Ahmed and Yang (2017) reported that supplementation with by-products of Punica granatum (fruit), which contains HT such as ellagitannins, punicalagin, punicalin, and pedunculagin, decreased the emission of ammonia and methanethiol from the excreta of broiler chickens. Moreover, a study by Bostami et al. (2015) demonstrated that supplementation of fermented pomegranate byproducts, containing ellagitannins, reduced gas emission (ammonia and hydrogen sulfide) from excreta in broiler chickens, potentially by reducing excreta microbial activity and pH. However, 2,000 mg/kg of chestnut wood tannic acid did not affect ammonia volatilization in broiler chickens. Hence, more research is required to identify other suitable types and concentrations of tannins to reduce nitrogen excretion and emissions of noxious and greenhouse gases during poultry production.

2.6.6. Effects of tannins on growth performance, immune system, gut microbiota, gut health, and meat quality in chickens raised under general conditions

While some authors showed that low concentrations (0.5 g/kg to 5 g/kg) of tannic acid improved growth performance (shown in Table 2.1.), other articles reported that supplementation of different sources of tannins at low concentrations (0.5 to 5 g/kg) did not affect growth performance (Cengiz et al., 2017; Mannelli et al., 2019; Rezar and Salobir, 2014) and even showed negative effects on growth performance of the birds (Jamroz et al., 2009). These differences may be a result of different tannin sources, supplementation period, or

specific experiment conditions (e.g., genetics of chickens, temperature, and abundance of pathogens in the living conditions).

Tannin supplementation at appropriate concentrations can improve immune response, gut health and gut microbiota of chickens raised under general conditions. Ramah et al. (2020) reported that whereas the high concentration of tannic acid (30 g/kg diet) can have a negative impact on immunity by decreasing the percentage of (cluster of differentiation) CD4+, CD8+, CD4+CD8+, and γδ+ cell populations in the thymus, spleen, and cecal tonsils as well as by reducing cytokine mRNA expression in spleen cells, concentrationlower concentrations of tannic acid (0.5 g/kg) enhanced CD4+CD8+ subpopulations and γδ+ cells in spleen and CD4+CD8+ subpopulations and B cells in cecal tonsils and increasing mRNA expression of IFN-γ in broiler chickens. A study by Kolodziej and Kiderlen (2005), reported that tannins were beneficial to maintain components of mucosal immunity of chickens via upregulating IgA and mucin 2. Erlejman et al. (2008) also demonstrated that CT can can bind to the tumor necrosis factor-α (a pro-inflammatory cytokine) receptor to inhibit inflammation, which implies that tannins directly modulate immune system without eliciting antimicrobial and antioxidant properties.

Diaz Carrasco et al. (2018) reported that a blend of chestnut (HT) and quebracho tannins (CT) altered the cecal microbiota of chickens and decreased genus Bacteroides and increased certain members of order Clostridiales predominately in the families Ruminococcaceae and Lachnospiraceae. A study by Koo and Nyachoti (2019) demonstrated that tannic acid positively affected microbial metabolites in pigs fed oxidized oil. Viveros et al. (2011) also suggested that tannins can have prebiotic effects via stimulating the proliferation of the beneficial bacteria. This microbiome modulating effect by tannins may partially explain

the gut health promoting effects of the chickens because microbiota of the chicken is closely associated with the gut health of chickens (Shang et al., 2018). Moreover, an *in vitro* study by Brus et al. (2018) reported that chestnut tannins stimulated proliferation of enterocytes and enhanced antioxidative properties of the chicken small intestinal epithelial cells. Bilić-Šobot et al. (2016) demonstrated that HT decreased production of cell debris in the large intestine of pigs, which lead to decreased production of skatole, which belongs to the indole family. Together, tannin supplementation has the potential to improve growth performance, gut microbiota, and gut health in broiler chickens, even in the absence of challenge models.

2.6. STRATEGIES TO MAXIMIZE THE EFFECTS OF SUPPLEMENTAL TANNINS IN CHICKENS

2.6.1. Heat process on tannins

Some *in vitro* studies have reported that heat processed HT had better antimicrobial and antioxidant properties than unprocessed HT (Ahmed and Yang, 2017; Ramah et al., 2020). This would be because heat processing could partially hydrolyze tannic acid and release gallic acid molecules, and these newly produced gallic acid and galloyl groups had enhanced antimicrobial and antioxidant effects compared to the fresh tannic acid (Kim et al., 2010). Kolodziej and Kiderlen (2005) also reported that thermal process of hamamelis virginiana containing gallotannins and CT improved efficacy of antioxidant properties for inhibiting lipid oxidation. However, because CT are hardly hydrolyzed, enhanced antioxidant of heat-processed hamamelis virginiana probably due to hydrolyzation of gallotannins in hamamelis virginiana rather than hydrolyzation of CT. Thus, while *in vitro* studies found that heat processing of tannins could improve their functional properties (e.g., antioxidant and antimicrobial effects) compared to unprocessed HT, it is unknown yet whether heat-processed

tannins have more beneficial effects on animal models.

2.6.2. Co-TA supplementation with other bioactive compounds

TA supplementation with other bioactive compounds could be more beneficial to chickens than supplementing tannins alone for several reasons: (1) interacted forms of tannins with proteins or polysaccharides may inhibit tannins to form a complex with endogenous and dietary proteins and minerals 2) distinct properties of bioactive compounds can show synergistic effects to antimicrobial effects against both gram negative and positive bacteria; (3) different bioactive compounds affect gut health in different ways, which can lead to synergistic effects in animals; and (4) by blending more than two bioactive compounds, pathogenic bacteria are less likely to thrive against diverse bioactive compounds. Table 2.3. demonstrates tannins with potential synergistic effects when combined with other bioactive compounds.

Probiotics are living microorganisms which beneficially affect the host animals by enhancing the animal's intestinal microbial balance (Adhikari and Kim, 2017). Probiotics may have different mode of actions from tannins to inhibit the growth of pathogenic bacteria and to improve gut health of chickens. Probiotics can improve gut integrity by modulating immunity and maintaining microflora of chickens and tannins, while tannins can show antioxidant and anti-inflammatory properties (Huyghebaert et al., 2011). However, one of the concerns of using probiotics with tannins could be that tannins may impair the probiotics. Interestingly, Khalil (2010) showed that gallic acid and catechin polyphenols did not inhibit the growth of Streptococcus thermophilus (probiotics), and Pacheco-Ordaz et al. (2018) reported that catechin, gallic, vanillic, ferulic and protocatechuic acids selectively inhibited the growth of pathogenic bacteria without decreasing viability of probiotics. Still, additional studies are required to confirm the synergistic effects and mechanisms of tannins and probiotics

in chickens.

Organic acids are organic compounds with acidic properties and known as strong antimicrobials. Tannins inhibit the growth of pathogens predominately by inhibiting activities of microbial enzymes and modulating bacterial membrane, but organic acids can penetrate the bacterial cell wall. These bacteria have to spend a lot of energy to pump out the hydrogen molecules, which causes bacterial death (Al-Harbi et al., 2018; Khan and Iqbal, 2016). Furthermore, organic acids are known to improve intestinal morphology and gut barrier integrity by serving as energy sources for epithelial cells, which may imply that organic acids with tannins can show synergistic effects (Chen et al., 2018a; Choi et al., 2020c; De Lange et al., 2010). Thus, a combination of tannins and organic acids can yield an increased synergistic antimicrobial and gut health promoting effect due to different mode of actions.

Table 2.3. Effects of tannins with other bioactive compounds on the chickens

Tannins	Other bioactive compounds	Outcomes	References
100 mg/kg tannic acid extract	Probiotics (1×10^4 spores/kg *Bacillus coagulans)*	Improved feed conversion ratio of coccidiosis vaccinated broilers.	(Tonda et al., 2018)
240 mg/kg tannic acid	Organic acids (420 mg/kg lactic, 480 mg/kg butyric acid and 480 mg/kg acetic acid)	Decreased *S. enteritis* horizontal transmission in broiler chickens	(Jarquin et al., 2007)
Chestnut tannins	Saturated short medium chain fatty acids (C4:0 to C12:0)	Showed strong antimicrobial effects in *in vitro* conditions and did not affect growth performance and meat quality of in chickens.	(Mannelli et al., 2019)

2.6.3. Supplementation of combined or encapsulated form of tannins

If tannins are combined with proteins, polysaccharides, and ions before being included in chicken feed, the tannins in complexes do not bind dietary and endogenous proteins and metal ions in chickens. These tannin complexes would dissociate in the high pH (>7.0) in the intestine of chickens, and proteins in the tannin complexes can be degraded by digestive enzymes in the small intestine of chickens (Barry et al., 1986; Gracia and de Castro, 1997). However, Lee et al. (2010) showed that supplementation of albumin-tannin complexes still decreased growth performance and negatively modulated microbiota, hematological indices and plasma iron status of weaning piglets. The delivery of tannin-protein or polysaccharide complexes in the GI tract of chickens, and the effects of diverse concentrations of supplemental tannin complexes on growth rate and gut health of chickens should be further investigated. The encapsulation process, which creates a physical barrier for bioactive compounds and separates the core material from the environment until their release, has generated a lot of attention in the livestock industry because encapsulation can maximize the efficacy of feed additives that have stability, cost, and environmental issues. Encapsulation has been applied to various vulnerable feed additives such as essential oils (Choi et al., 2020b; Omonijo et al., 2018a; Yang et al., 2020), probiotics (Liu et al., 2015), organic acids (Choi, 2019), bacteriophages (Tang et al., 2013), zinc (Jang et al., 2014) and exogenous enzymes (Liu et al., 2017b). Diverse materials including proteins (Yang et al., 2020; Zhang et al., 2014), lipids (Choi, 2019), carbohydrates (starch) (Omonijo et al., 2018a), and polysaccharides (Tang et al., 2013; Trabelsi et al., 2016) have been used to encapsulate bioactive compounds.

Encapsulation techniques can be applied to decrease side effects and maximize benefits

of tannins in chickens. Encapsulation of tannins can depress the protein binding capacity of tannins, which decreases feed intake by making astringent taste and digestibility of proteins and induces dietary and endogenous protein losses (Fernández et al., 2012). In addition, a larger concentration of tannins can be delivered to the small or large intestine where many pathogens propagate by decreasing bioavailability for absorption in the upper GI tract of chickens. Adejoro et al. (2019) showed that lipid-encapsulated acacia tannin extracts reduced methane production and enhanced neutral detergent fiber digestibility in sheep. A study by Wang et al. (2020b) reported that microencapsulated tannic acid improved intestinal morphology in duodenum, increased expression of ileal nutrient transporters (sodium-dependent neutral amino acid transporter; B0AT1 and peptide transporter 1; PepT1) and altered the microbiota without affecting growth performance of weaned piglets even though ileal maltase activity and gene expression of jejunal sodium-dependent glucose transporter 1 (SGLT1) was reduced. Future studies are required to develop effective encapsulated tannins and to determine appropriate concentrations of encapsulated tannins for chickens.

2.7. CONCLUSION

There are various kinds of tannins, which have different bioavailability in chickens. Depending on the type and concentration of tannins employed as afeed additive, the effects on growth performance and gut health of the broiler chickens can be beneficial or detrimental. In addition, heat processing, co-supplementation with other bioactive compounds, and encapsulation potentially may enhance the beneficial biological effects of tannins. In conclusion, TA supplementation alone or in conjunction with those strategies has the potential to alleviate challenges, replace AGP and improve production efficiency in poultry productions.

3.0. CHAPTER 3 MANUSCRIPT I

IN VITRO AND IN VIVO EVALUATION OF TANNIC ACID AS AN ANTIBACTERIAL AGENT IN BROILERS INFECTED WITH SALMONELLA TYPHIMURIUM[1]

[1]**Choi, J.**, S. Yadav, S. Vaddu, H. Thippareddi, W. K. Kim. In vitro and in vivo evaluation of tannic acid as an antibacterial agent in broilers infected with Salmonella Typhimurium. Submitted to Poultry Science.

3.1. ABSTRACT

This study was conducted to evaluate tannic acid (TA) as an antibacterial agent against *Salmonella* Typhimurium in *in vitro* and *in vivo* chicken model. The TA formed an inhibitory zone against *Salmonella enterica* serotypes including *S.* Typhimurium, *S.* Enteritidis, *S.* Infantis. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of TA against *Salmonella* Typhimurium nalidixic acid resistant strain (ST^{NR}) was determined as 40 and 700 μg/mL, respectively. Sub-lethal doses of TA (5, 10, and 20 μg/mL) restricted swimming and swarming motility and biofilm formation of ST^{NR} compared to the control group (0 μg/mL) ($P < 0.05$). The TA-BSA complex formed at simulated gastric pH (pH 3.75) were hydrolyzed at pH 6.75 and 7.25 ($P < 0.05$), and the hydrolysis of TA-BSA complex was stronger at pH 7.25 compared to the pH 6.75 ($P < 0.05$). The inhibitory zone of TA-BSA complex against ST^{NR} at pH 6.75 was lower than TA without BSA at 30 and 60 min ($P < 0.05$), but not at 120 min ($P > 0.1$). The inhibitory zone of TA-BSA complex against ST^{NR} at pH 7.25 was not decreased at 0, 30, and 60 min compared to TA without BSA ($P > 0.1$). The recovery rate of TA was 83%, 54.8%, 10.5%, and 19.6% in the gizzard, jejunum, ileum, and ceca, respectively in chickens. The ST^{NR} infected broilers fed 0.25 g/kg of TA had significantly lower distance to own group compared to the sham-challenged control (SCC) and challenged controlled (CC) group on D 21. TA supplementation linearly ($P < 0.05$) and quadratically (tendency; $P = 0.071$) reduced relative abundance of the family Peptostreptococcaceae in broilers infected with ST^{NR} on D 7. TA supplementation linearly ($P < 0.05$) and quadratically (tendency; $P = 0.06$) increased the relative abundance of

the family Erysipelotrichaceae in broilers infected with ST^{NR} on D 21. Therefore, TA has potential to be used as an antibacterial agent against the *S.* Typhimurium in broilers.

Keywords: Tannic acid, *Salmonella* Typhimurium, antibacterial agent, recovery rate, microbiome, broiler chickens.

3.2. INTRODUCTION

Symptoms of salmonellosis (e.g., infection *Salmonella* spp.) include diarrhea, fever, and even mortality in humans (Sanchez et al., 2002). According to CDC (2021), reported cases of salmonellosis in the US alone are 1.35 million people with 26,500 hospitalizations and 420 deaths every year. *Salmonella enterica* serovar Typhimurium is one the most common serotypes in poultry production (Kaiser et al., 2000). Most of chickens infected with *S.* Typhimurium are asymptomatic carriers, which can effectively spread *S.* Typhimurium in a flock or in food contamination (Kogut and Arsenault, 2017). Still, *S.* Typhimurium can induce acute gastroenteritis and bacteremia, which stimulate immune system and decrease growth performance by colonizing and invading the gastrointestinal tract of chickens (Griffin and McSorley, 2011).

In the past, antibiotics at therapeutic and sub-therapeutic concentrations have been supplemented in poultry diets to control *S. Typhimurium* infection and to improve growth performance and gut health of chickens (Chambers and Gong, 2011). The use of antibiotics has been banned or restricted in many countries because the continuous use of antibiotics can cause generation of antibiotic resistant bacteria (Castillo, 2012). There are already many antibiotic resistant strains of *Salmonella* spp. such as tetracycline, ciprofloxacin, ampicillin, etc., and actions are required to control the spread and infection of antibiotic resistant strains of *Salmonella* spp. (Yuan and Guo, 2017). Furthermore, some producers in the U.S. poultry industry are determined to raise chickens with a "no antibiotics ever (NAE)" or "raised without antibiotics (RWA)" approach (Singer et al., 2019). Thus, it is essential to find appropriate

bioactive antibacterial agents to control *Salmonella* infection in the poultry industry (Yang et al., 2015a).

Tannic acid (TA), considered as the standard of hydrolysable tannins, are polyphenolic compounds that can interact with proteins (Hagerman and Butler, 1978). The TA has a glucose as a polyol (central core), which is esterified with 9 to 10 gallic acid (GA) molecules, and TA can be hydrolyzed by heat, tannase, and low and high pH as their names refer (Kim et al., 2010). Whereas tannins are known to exhibit antibacterial effects and anti-virulence effects against diverse pathogens in *in vitro* condition (Kim et al., 2010; O'May et al., 2012), antibacterial and anti-virulence of TA against *S.* Typhimurium in chickens is still uncertain. Interaction of TA with dietary and endogenous nutrients can restrict the availability of the hydroxyl groups of TA in the gastrointestinal tract of chickens (Zhao et al., 1997). In addition, whether dietary TA is delivered to the lower gastrointestinal tract, where *S.* Typhimurium and many other pathogenic bacteria inhabit, has not been elucidated yet. Therefore, the purposes of the study were 1) to investigate antibacterial and anti-virulence effects of TA against *S.* Typhimurium in *in vitro* chicken model; 2) to investigate antimicrobial effects of heat processed TA; 3) to elucidate the recovery rate of TA in the different part of the gastrointestinal tract of chickens; and 4) to investigate effects of dietary TA on ileal microbiota of chickens infected with *S.* Typhimurium.

3.3. MATERIALS AND METHODS

3.3.1. Inhibitory zone assay of tannic acid and gallic acid against *Salmonella enterica* serotypes

An agar well diffusion method was performed to check antibacterial effects of TA (ACS reagent, Sigma-Aldrich, St. Louis, MO) and GA (ACS reagent, Sigma Aldrich) against

diverse *Salmonella enterica* serotypes according to Balouiri et al. (2016) and Hudzicki (2009) with some modifications. The inoculum of each *Salmonella* strain was cultured in trypticase soy broth (TSB; Sigma Aldrich) with designated antibiotics overnight as described in Table 3.1. Next day, 1×10^8 CFU/mL of *Salmonella* inoculum was made by adding peptone water (0.1%; Sigma Aldrich) from the overnight culture by using OD600. Afterwards, freshly prepared trypticase soy agar (TSA) plate (82 × 15 mm petri dish containing 15 mL TSA) was inoculated with the *Salmonella* inoculum using a swab. The TSA plate was aseptically punched with a sterile cork borer (6 mm diameter). One drop of soft TSB agar (containing 1.1% of agar) was added into the punched hole. Fifty microliters of 10 μg/mL TA and GA in distilled water and in a 5% dimethyl sulfoxide (DMSO, Sigma Aldrich) solution, respectively, were added to the punched hole in the TSA plate and placed at 4°C for 3 h to stimulate diffusion. Afterwards, the agar plates were incubated at 35°C for 24 h, and the diameter of an inhibitory zone was measured by using a CD5APXR digital caliper (Absolute AOS Digimatic, Mitutoyo Corporation, Kanagawa, Japan). This assay included independent 3 replicates with 2 technical replicates.

3.3.2. Minimum inhibitory concentration and minimum bactericidal concentration of tannic acid and tannic acid against *Salmonella* Typhimurium nalidixic acid resistant strain, and growth curve of tannic acid against *Salmonella* Typhimurium nalidixic acid resistant strain

The minimum inhibitory concentration (MIC) of TA against ST^{NR} was determined using the microdilution method by measuring visual turbidity according to Carretto et al. (2018) and measuring OD600 according to Van Parys et al. (2010) and Wiegand et al. (2008). Different concentrations of TA (10 to 2,000 μg/mL) and GA (1 to 10 mg/mL) were freshly

prepared in distilled water and 5% of dimethyl sulfoxide (DMSO), respectively. The 50 µL of dilutions of test compounds were added to each well in the 96 well plate (round bottom), and 50 µL of *Salmonella* suspension (1×10^6 CFU/mL in the fresh TSB solution) was added to obtain desired test compound concentrations and 5×10^5 CFU/mL as the final desired inoculum. Each row included growth control (50 µL of 1×10^6 CFU/mL and 50 µL of distilled water) and sterile control (100 µL of TSB). After plating growth controls, 10 µL from each growth control was serially diluted with peptone water to determine inoculated *Salmonella* concentration. The 96 well plate was incubated at 35°C for 16 to 20 h. The MIC of TA and GA were determined as the lowest concentration entirely inhibited the bacterial growth in the wells by measuring visual turbidity using a microtiter mirror (Sensi-touch, Sensititre, Westlake, OH) and by measuring OD600 (Carretto et al., 2018; Van Parys et al., 2010). By using OD600 values of each well, sub-lethal concentrations of TA that did not decrease viability of ST^{NR} were determined and used for swimming and swarming motility and biofilm assays.

After determining MIC concentrations of TA and GA, 10 µL from wells of higher concentrations than MIC was plated on BGS agar containing 200 µg/mL nalidixic acid sodium salt (NA; Sigma Aldrich) and incubated 24 h to determine minimum bactericidal concentration (MBC). The MBC is defined as the lowest concentration of antibacterial effect that decreases the viability of the initial bacterial inoculum by ≥99.9% (3 log decrease) (Alade and Irobi, 1993). The MIC and MBC levels were confirmed by conducting three independent assays with two technical replicates.

3.3.3. Effects of simulated pelleting heat and autoclave heat on antimicrobial effects and protein precipitation capacity of TA

Antimicrobial effects of TA exposed to simulated pelleting heat were evaluated according to Liu et al. (2017b) with some modifications. One gram of TA was added in a 15 mL plastic tube. In a preheated (40, 50, 60, 70, 80 and 90°C) water bath (Isotemp 3013, Fisher Scientific; Pittsburgh, PA), the sample tube was incubated for 30 min with a loosen lid, and at 15 min the tube. Afterwards, the heat-processed TA was stored at room temperature and for future analyses.

To determine effects of autoclave heat and steam on the antimicrobial effects of TA, one gram of TA was added in 15 mL tube with a loosely closed lid and autoclaved (121°C) for 5, 10, 15, 20, 30 and 60 min using an autoclave (Market Forge Industries; 6551 Jansen Avenue, Albertville, MN). To minimize heat variation, all samples were removed from the autoclave after having 5 min of the cooling process. Afterwards, the heat-processed TA was stored at room temperature and for further analyses.

Antimicrobial effects of the heat-processed TA were evaluated by using the inhibitory zone assay as described above. The test compounds were dissolved in distilled water (10 mg/mL) and added into each well. To determine precipitated TA with BSA, 2.5 mg of fresh or the processed TA and 5 mg of BSA were added into the 50 mL of the solution of distilled water at pH 4.5 to 5.0. The protein precipitation capacity of the processed TA was compared to the fresh TA. This assay included independent three replicates.

3.3.4. Swimming and swarming motility and biofilm assays of tannic acid against ST^{NR}

Swimming and swarming motility of ST^{NR} when exposed to sub-MIC levels of TA were evaluated in Petri dishes (polystyrene, diameter of 82 mm) containing swim agar [nutrient broth (NB, Sigma Aldrich), 0.25% bacteriological agar (Sigma Aldrich), and 0.5% glucose (Sigma Aldrich)] and swarm agar (NB, 0.25% bacteriological agar, and 0.5% glucose), respectively, according to Chelvam et al. (2014) with some modifications. The plates were dried with the lid closed for 50 min and dried with lid open for 10 min. Afterwards, plates were inoculated with 10^8 CFU/mL ST^{NR} using a sterile toothpick. After incubation at 35°C for 24 h, the relative swimming and swarming area compared to the agar plate size were determined by using ImageJ (National Institutes of Health, Bethesda, MD). This assay included three independent replicates, and the swimming and swarming motility values of ST^{NR} in the swimming and swarming plates containing TA (5, 10, and 20 μg/mL) were expressed relative to the control (0 μg/mL).

Anti-biofilm formation activity of TA were determined according to Crawford et al. (2008) with some modifications. Overnight culture (10^9 CFU/mL) of ST^{NR} was serially diluted to 10^6 CFU/mL by adding fresh TSB. In glass tubes, 1.5 mL of 10^6 CFU/mL of ST^{NR} and 1.5 mL of TA solutions (0, 10, 20 and 40 μg/mL) were added to make 5×10^5 and 0, 5, 10, and 20 μg/mL, respectively. Afterwards, the cultures were incubated at 35°C for 48 h and then gently washed three times by adding phosphate buffered saline (PBS) to remove planktonic bacteria. The samples were incubated at 60°C for 1 h for fixation. A solution of 0.1% crystal violet [gentian violet in isopropanol-methanol-PBS (1:1:18)] was added to stain cells for 30 min at room temperature. Afterwards, undyed crystal violet was removed by using PBS until the

liquid ran clear. Finally, the dye was extracted by adding 33% acetic acid, vortexed, and then quantified with optical density readings at 570 nm to determine the amount of dye retained by biofilm cells.

3.3.5. Dynamics of protein precipitation capacity and antibacterial effects of tannic acid-bovine serum albumin complex formed in simulated gastric pH (pH 3.75) when exposed to simulated intestinal pH (pH 6.75 or pH 7.25)

To simulate the formation of TA-BSA complex in gastric pH, 50 mg TA and 100 mg BSA were added in 10 mL of distilled water (pH 3.75) in three tubes. Afterwards, one tube was used to represent simulated gastric pH, and pH levels of the other two tubes were modulated to pH 6.75 and pH 7.25, respectively, by using HCl (0.1 mol/L) and NaOH (0.1 mol/L). The same volume of distilled water (pH 3.75) was added to the tube representing simulated gastric pH. After adjusting pH and vortexing, 200 μL and 100 μL of the solution were sampled from each tube to determine antibacterial effects and precipitated TA, respectively, to represent the 0 min time point. The two tubes were shaken at 200 rpm for 30, 60, and 120 min at 39°C in the MaxQ™ 4000 Incubator Shaker (Thermo Scientific, Marietta, OH). Sampling was conducted at each time point (30, 60, and 120 min), and the pH level of the solutions were maintained at pH 6.75 and pH 7.25 during the incubation. Immediately after sampling, the samples for determining precipitated TA were centrifuged for 15 min at 5,000 × *g* at room temperature, and the supernatants were thoroughly removed and dried. The concentration of TA in the precipitate (TA-BSA complex) was analyzed according to Hagerman and Butler (1978). The TA-BSA precipitate was dissolved in 4 mL of the SDS-triethanolamine solution which contained 1% (v/v) SDS and 5% (v/v) triethanolamine in distilled water and vortexed to

hydrolyze TA-BSA complex. One milliliter of the ferric chloride reagent (0.01 mol/L ferric chloride in 0.01 mol/L HCl) was added and mixed immediately. After 30 min, the samples were transferred to a 96 well plate, and the absorbance at 510 nm (Waterhouse, 2002) was read for TA in triplicate, respectively. The average of triplicate samples were used to calculate the TA concentration in the sodium dodecyl sulfate (SDS)-triethanolamine and ferric chloride solution with prepared standard curve. The 50 μL of vortexed samples was immediately added to the prepared agar well for the inhibitory zone assay. Precipitated TA at different pH levels and different time points was compared to sample at simulated gastric pH, and antibacterial effects were compared to the 5 mg/mL TA without BSA. This assay included three independent replicates with two technical replicates.

3.3.6. Recovery rate of tannic acid in the different part of the gastrointestinal tract of chickens

The current study was reviewed and approved by Institutional Animal Care and Use Committee at the University of Georgia, Athens, GA. A total of one hundred 28-day-old Cobb 500 male broilers were allocated to 4 replicates of 25 birds per pen. The experimental diets were formulated to meet or exceed requirement levels of energy and nutrient based on the Cobb Broiler Management Guide (Cobb 2018) and contained 3 g/kg titanium dioxide as an inert marker and 2 g/kg TA. During the experimental period (D 28 to 35), birds had free access to feed and water. On D 35, 4 birds per pen were euthanized via cervical dislocation, and contents of gizzard, jejunum, ileum, and ceca were collected in sample bags. Feed and digesta samples were oven-dried at 75°C till constant weight. The concentrations of titanium dioxide in the feed and digesta samples were determined according to the method of Short et al. (1996). The concentration of TA in the feed and digesta samples were analyzed by Creative Proteomics

(Shirley, NY). The 0.1 g of feed and digesta samples were mixed with 1 mL of N, N-Dimethylformamide in 2 mL tubes and vortexed for 90 s and shaken for 60 min. Samples were centrifuged at 4,000 × *g* at 4°C for 10 min. Afterwards, 0.25 mL supernatant was mixed with 1.5 mL D.D. water and 0.25 mL NH_4OH for baseline (B), and another 0.25 mL supernatant was mixed with 1.25 mL D.D. water, 0.25 mL NH_4OH, 0.25 mL ammonium ferric citrate solutions and kept for 10 min (A). Absorbance at 525 nm was determined, and the net absorbance value (A – B) was used to quantify TA concentration in the feed or digesta by using the prepared standard curve. The recovery rate of TA in the different segments including gizzard, jejunum, ileum, and ceca of the gastrointestinal tract of chickens was calculated as follows:

$$Tannic\ acid_{RECOVERY}\ (\%) = \left[\frac{(Marker_{DIET} \times Tannic\ acid_{DIGESTA})}{(Tannic\ acid_{DIET} \times Marker_{DIGESTA})}\right] \times 100$$

3.3.7. Evaluation of effects of tannic acid on ileal microbiome of chickens infected with *Salmonella* Typhimurium nalidixic acid resistant strain

The current study was reviewed and approved by Institutional Animal Care and Use Committee at the University of Georgia, Athens, GA. A total of 546 one-day-old male broilers was randomly allocated to 6 experimental groups, and each group had 7 replicates with 13 birds per cage. The treatment groups were 1) Sham-challenged control (SCC; birds fed a basal diet and administrated peptone water); 2) Challenged control (CC; birds fed a basal diet and inoculated with 10^8 ST^{NR}); 3) Tannic acid 0.25 (TA0.25; CC + 0.25 g/kg TA); 4) Tannic acid 0.5 (TA0.5; CC + 0.5 g/kg TA); 5) Tannic acid 1 (TA1; CC + 1 g/kg TA); and 6) Tannic acid 2 (TA2; CC + 2 g/kg TA). Basal diets (starter: D 0 to 7 and grower: D 7 to 21) were formulated to meet or exceed energy according to Cobb Broiler Management Guide (Cobb 2018). On D

0, the SCC group and ST^{NR} infected groups were administrated with 0.5 mL peptone water and 0.5 mL of 10^8 ST^{NR} in peptone water, respectively. On D 7 and 21, one bird per pen was euthanized via cervical dislocation, and mid-ileal content was collected and snap-frozen in liquid nitrogen. Samples were stored at – 80°C for further analysis.

DNA was extracted from the contents of the mid-ileum by utilizing QIAamp® PowerFecal® DNA Kit (Qiagen, Hilden, Germany) according to manufacturer procedure. The quantity and quality of extracted DNA were confirmed by using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). The 16s rRNA gene sequencing was assisted by LC sciences, LLC (Houston, TX). The V3 and V4 regions were amplified the primers of 338F (5-CCTACGGGNGGCWGCAG-3)/806R (5-GACTACHVGGGTATCTAATCC-3) with PCR procedures as follows. The 5' ends of the primers were labelled with specific barcodes per sample and sequencing universal primers. The PCR amplification was performed in a total volume of 25 µL reaction mixture containing 25 ng of template DNA, 12.5 µL PCR Premix, 2.5 µL of each primer, and PCR-grade water to adjust the volume. The PCR amplification program of the prokaryotic 16S fragments were an initial denaturation at 98°C for 30 s; 32 cycles of denaturation at 98°C for 10 s, annealing at 54°C for 30 s, and extension at 72°C for 45 s; and then final extension at 72°C for 10 min. The PCR products were checked using 2% agarose gel electrophoresis. The PCR products were purified using AMPure XT beads (Beckman Coulter Genomics, Danvers, MA) and quantified by Qubit (Invitrogen, Carlsbad, CA). For sequencing, the amplicon pools were prepared, and the size and quantity of the amplicon library were measured using Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA) and the Library Quantification Kit for Illumina (Kapa Biosciences, Woburn, MA),

respectively. The libraries were sequenced using the NovaSeq PE250 platform. QIIME2 (version 2022.02) was used to analyze sequenced data (Bolyen et al., 2019). Sequences were demultiplexed and denoised by employing demux emp-paired function and QIIME2 plugin DADA2, respectively. Alpha diversity and beta diversity were analyzed by QIIME2's built in functions. A phylogenetic tree of ASV was created, and the taxonomy of each ASV was classified by using a pre-trained classifier the reference Greengenes reference database, and phylum- and family-level composition were shown.

3.3.8. Statistical analyses

Statistical analyses and graph construction were performed using GraphPad Prism software version 9.1.0 (GRAPH PAD software INC, CA). To obtain the concentrations that did not affect growth of the bacteria, the OD600 value of each concentration was analyzed using one-way ANOVA followed by a Dunnett's post-hoc test (Control: 0 μg/mL). For studying *in vitro* hydrolyzation of TA-BSA at simulated intestinal pH, precipitated TA at the intestinal pH solution were expressed as relative to precipitated TA at simulated gastric pH and analyzed using one-way ANOVA followed by a Dunnett's post-hoc test (Control: precipitated TA at simulated gastric pH). The inhibitory zone diameters of the simulated gastric and intestinal solutions were shown as relative to the inhibitory zone diameters of pure TA without BSA and analyzed using one-way ANOVA followed by a Dunnett's post-hoc test (Control: pure TA without BSA). In addition, unpaired t-test was used to compare precipitated TA and the inhibitory zone diameter between pH 6.75 and 7.25. Swimming and swarming motility and biofilm data were expressed relative to control and compared using one-way ANOVA followed by Dunnett's post-hoc test (control: 0 μg/mL).

For microbiome analyses, statistical analyses were performed using SAS (version 9.4; SAS Inst. Inc., Cary, NC). The effects of ST^{NR} inoculation (SCC vs. CC) were evaluated by the unpaired t-test. Challenged groups (CC, TA0.25, TA0.5, TA1, and TA2) were compared using PROC MIXED in a completely randomized design followed by the Tukey's comparison test. Orthogonal polynomial contrasts were utilized to evaluate the significance of linear and quadratic effects of different concentrations of TA within the treatments infected with ST^{NR}. For the beta diversity indice (unweighted unifrac) of the cecal microbial communities, each treatment group was set as the control group, and one-way ANOVA with Dunnett's post hoc test was used, and statistical significance was set at $P < 0.05$, and trends ($0.05 \leq P \leq 0.1$) were also presented.

3.4. RESULTS

3.4.1. Antibacterial effects of tannic acid and gallic acid against *Salmonella enterica* serotypes in *in vitro* conditions

The averages of the inhibitory zone diameters of 10 μg/mL TA and GA against diverse *Salmonella enterica* serotypes are shown in Table 3.1. The TA formed an inhibitory zone against diverse *Salmonella* spp. including *S.* Typhimurium, *S.* Enteritidis, and *S.* Infantis, but GA did not form an inhibitory zone against the *Salmonella enterica* serotypes.

Table 3.1. Diameter (mm) of an inhibitory zone of 10 mg/mL tannic acid and gallic acid against *Salmonella enterica* serotypes [1]

Strains	Added antibiotics	Sources	Diameter (mm) of inhibitory zone against 10 mg/mL TA
S. Typhimurium nalidixic acid resistant strain (ST^{NR})	200 µg/mL nalidixic acid	Yadav et al. (2022a)	18.65 ± 0.22
S. Typhimurium nalidixic acid and novobiocin resistant strain	20 µg/mL nalidixic acid and 25 µg/mL novobiocin	ATCC BAA-184	18.78 ± 0.1
S. Enteritidis nalidixic acid resistant strain	25 µg/mL nalidixic acid	Shanmugasundaram et al. (2021)	17.91 ± 0.2
S. Enteritidis wild type	N/A	Luoma et al. (2017)	17.88 ± 0.32
S. Enteritis nalidixic acid resistant strain	200 µg/mL nalidixic acid	Adhikari et al. (2018)	18.73 ± 0.05
S. Infantis	N/A	ATCC DUP-103	20.26 ± 0.22

[1] 10 mg/mL GA did not form an inhibitory zone against all *Salmonella enterica* serotypes.

[2] Value was shown as mean ± SEM, $n = 3$.

By measuring visual turbidity and using OD600 measurement, the MIC of TA against ST^{NR} was determined as 40 μg/mL (Figure 3.1.A and 3.2.), and the MBC of TA against ST^{NR} was determined as 700 μg/mL. The MIC of GA against ST^{NR} was determined as 8,000 μg/mL (Figure 3.1B.), and the MBC was higher than 10,000 μg/mL. Growth curve of ST^{NR} exposed to different concentrations of TA by using OD600 to determine sub-lethal concentrations of TA was shown in Figure 3.2., and 10 μg/mL and 5 μg/mL of TA were statistically similar with the growth control ($P > 0.05$).

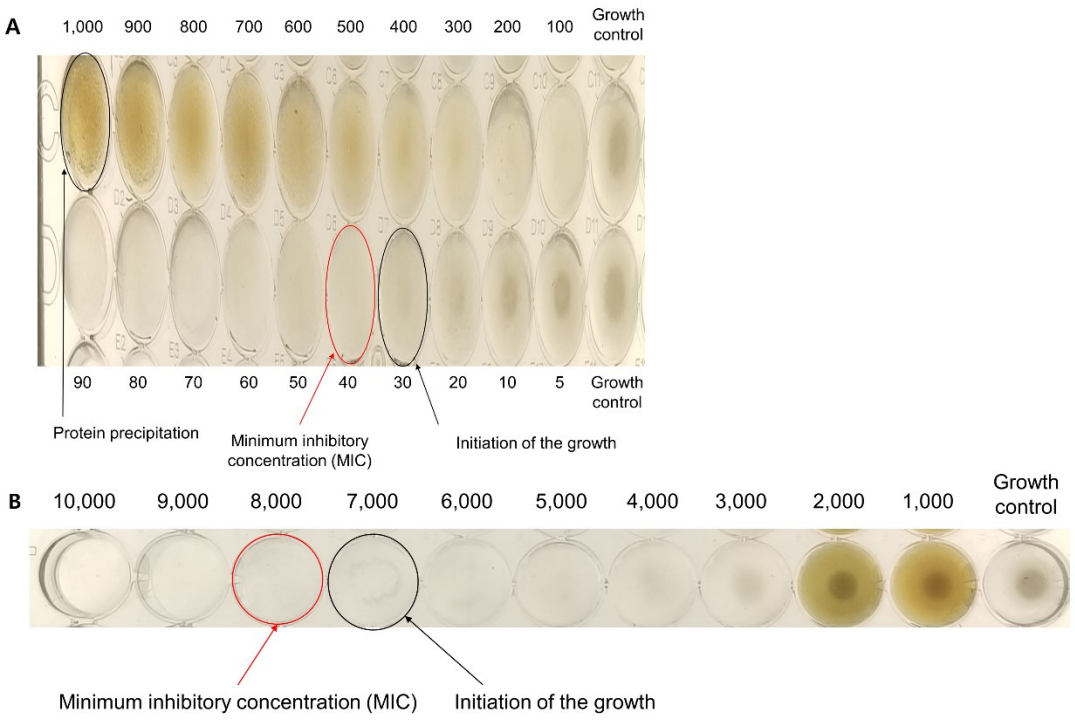


Figure 3.1. Visual turbidity measurement to determine minimum inhibitory concentration (MIC) of tannic acid (A) and gallic acid (B) against *Salmonella* Typhimurium nalidixic acid resistant strain. Different concentrations of tannic acid (1,000 to 5 µg/mL) or gallic acid (10,000 to 1,000 µg/mL) and *Salmonella* inoculum (3 to 4 × 10^5) in tryptic soy broth containing nalidixic acid (200 µg/mL) was incubated at 35°C for 20 hours.

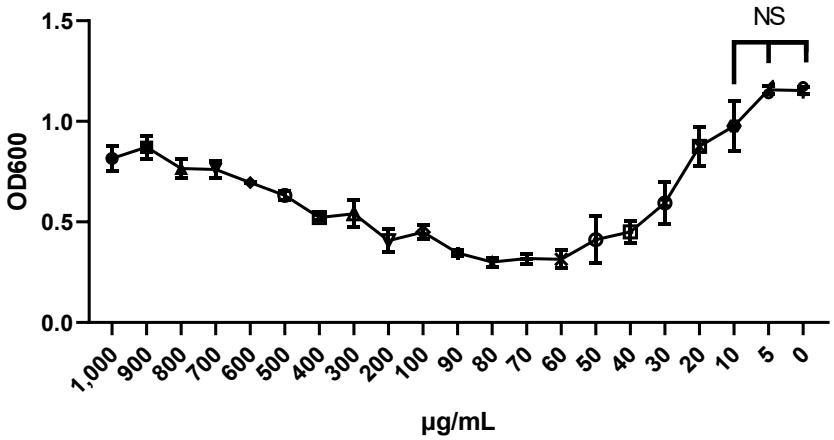


Figure 3.2. Growth curve of *Salmonella* Typhimurium nalidixic acid resistant strain cultured in tryptic soy broth with diverse concentrations of tannic acid (0 to 1,000 µg/mL). The NS means not significantly different ($P > 0.1$) according to one-way ANOVA with Dunnett's post hoc test (control: 0 µg/mL tannic acid). Each value represents means ± SEM, $n = 3$.

3.4.2. Effects of simulated pelleting heat and autoclave heat on antimicrobial effects and the protein precipitation capacity of TA

As described in Figure 3.3., at 60 and 70°C, the inhibitory zone diameter against ST^{NR} was significantly higher than the non-processed TA ($P < 0.05$). Autoclave processing for 5, 10, 15, 20, 40 and 60 min did not differ the inhibitory zone diameter against ST^{NR} compared to the unprocessed TA ($P > 0.05$). The protein precipitation capacity of TA was not modulated when they were exposed to the simulated pelleting heat and autoclave heat ($P > 0.05$).

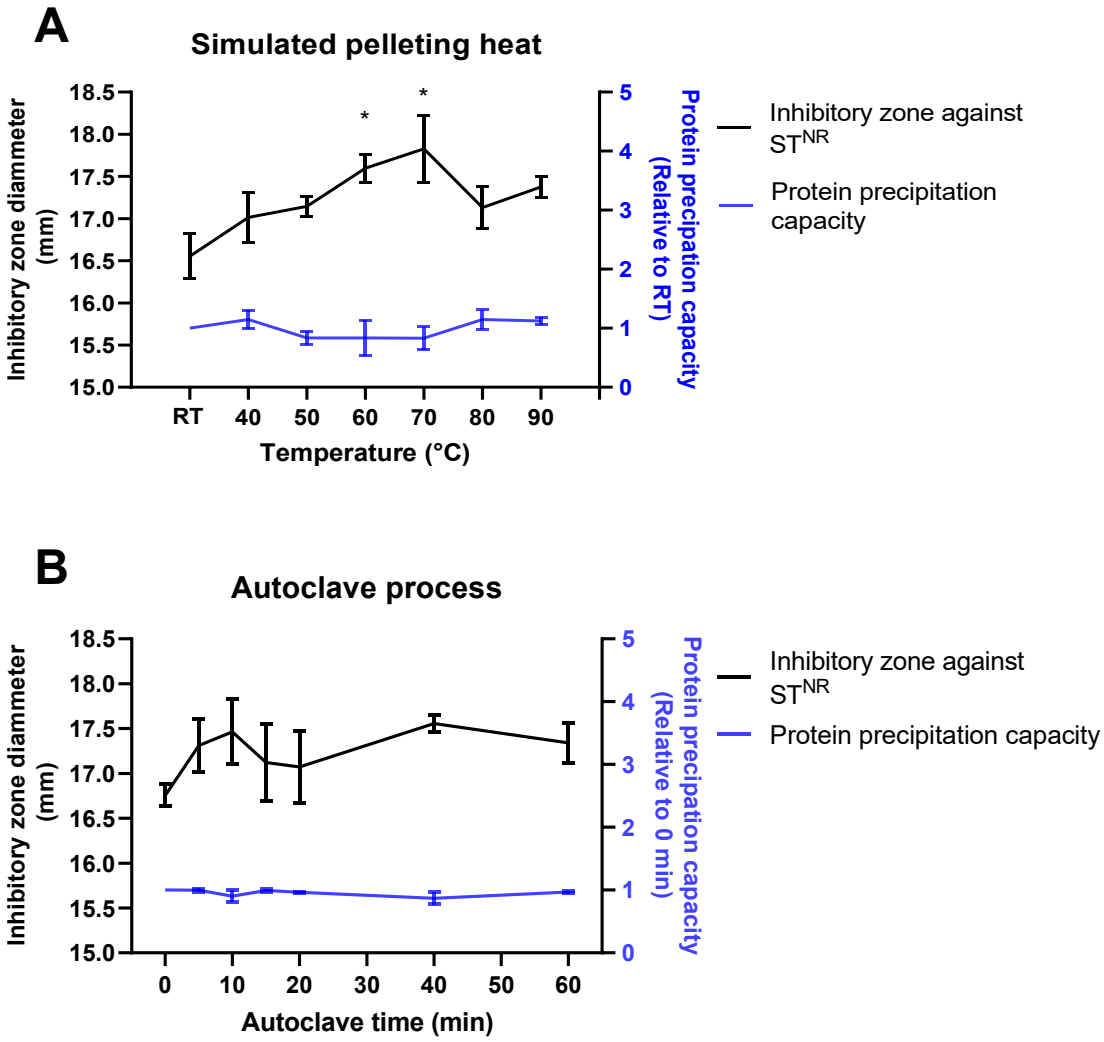


Figure 3.3. Effects of simulated pelleting temperatures (A) and autoclave heat (B; 121°C) on the inhibitory zone diameter against *Salmonella* Typhimurium nalidixic acid resistant (ST^{NR}) strain and protein precipitation capacity of tannic acid. Data were shown as mean + SEM. The * denotes significant difference ($P < 0.05$) according to one-way ANOVA with Dunnett's post hoc test (control: unprocessed tannic acid).

3.4.3. Effects of sub-lethal levels of tannic acid on swimming and swarming motility and biofilm formation of *Salmonella* Typhimurium nalidixic acid resistant strain

The ST^{NR} without TA exhibited both swimming and swarming motility. As shown in Figure 3.4., when the sub-lethal doses (5, 10, and 20 μg/mL) of TA were added in the swimming and swarming plates, the swimming and swarming motility of ST^{NR} were restricted compared to the control ($P < 0.05$).

In TSB, ST^{NR} formed biofilm at 48 h at 35°C. As shown in Figure 3.4., the biofilm formation of ST^{NR} was significantly inhibited due to the sub-lethal concentrations of TA (5, 10, and 20 μg/mL) compared to the control.

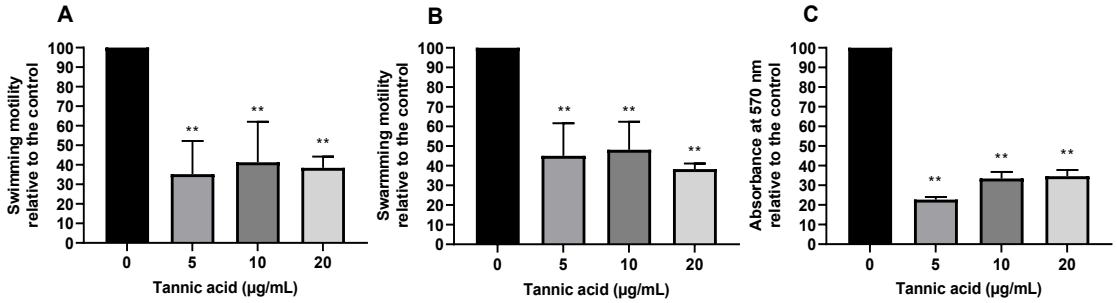


Figure 3.4. Swimming (A) and swarming (B) motility (covered area of agar plate) and biofilm formation activity (C) of *Salmonella* Typhimurium nalidixic acid resistant strain when exposed to sub-lethal concentrations (5, 10 and 20 µg/mL) of tannic acid relative to the control (100%). The ** denotes a significant difference ($P < 0.05$) compared to the control according to one-way ANOVA with Dunnett's post hoc test (control: 0 µg/mL). Each value represents means ± SEM.

3.4.4. Dynamics and antibacterial effects of tannic acid-bovine serum albumin complex formed in simulated gastric pH when exposed to simulated intestinal tract pH

As shown in Figure 3.5., there was significantly lower amount of TA-BSA complex at all time points (0, 30, 60, and 120 min) when TA-BSA complex formed at simulated gastric pH was subjected to the simulated intestinal pH 6.75 and 7.25 compared to the simulated gastric pH ($P < 0.05$). The pH 7.25 solution more hydrolyzed TA-BSA complex formed at gastric pH compared to the pH 6.75 solution ($P < 0.05$).

Antibacterial effects of TA-BSA complex at simulated gastric pH were lower than pure TA without BSA ($P < 0.05$). The TA-BSA complex in the pH 6.75 solution at 30 min ($P = 0.052$) tended to have a lower inhibitory zone diameter compared to the pure TA without BSA. The TA-BSA complex in the pH 6.75 solution at 30 min and 60 min had a significantly lower inhibitory zone diameter compared to the pure TA without BSA. However, TA-BSA complex in the pH 6.75 solution at 120 min had a statistically similar inhibitory zone compared to the pure TA ($P > 0.1$). The inhibitory zone of the TA-BSA solution at pH 7.25 at 0, 30, and 60 min was statistically similar to the pure TA ($P > 0.1$). However, the inhibitory zone of the solution at 120 min was significantly lower than the pure TA ($P < 0.05$). The TA-BSA solution at pH 7.25 solution had a greater inhibitory zone compared to the TA-BSA solution at pH 6.75 ($P < 0.05$).

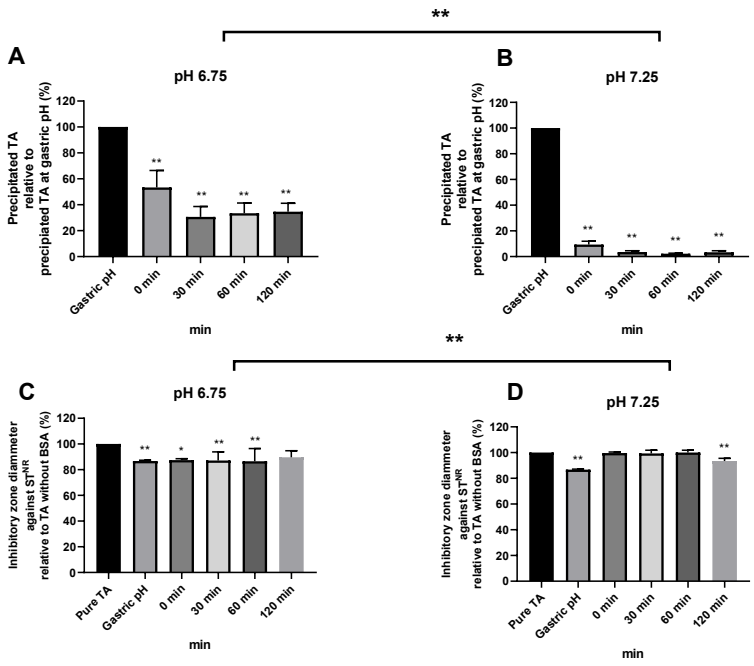


Figure 3.5. Dynamics of the protein precipitation capacity (A and B) and antibacterial effects (C and D) of tannic acid-bovine serum albumin formed in simulated gastric pH (pH 3.75) when subjected to simulated intestinal pH (pH 6.75 or pH 7.25). Within the same pH, one-way ANOVA with Dunnett's post hoc test (control of A and B: precipitated TA in simulated gastric pH; control of C and D: the inhibitory zone diameter of pure TA without BSA) was used. Student t-test was used to compared precipitated TA and the inhibitory zone diameter between pH 6.75 and 7.25. The * denotes a tendency ($0.05 \leq P \leq 0.10$) and ** denotes a significance ($P < 0.05$). Each value represents means ± SEM, $n = 3$.

3.4.5. Intestinal delivery of tannic acid in the different part of the gastrointestinal tract of chickens

Figure 3.6. shows the recovery rate of TA in the gastrointestinal tract of chickens. In the gizzard and jejunum, 83% and 54.8% of TA was recovered, respectively. In the ileum, 10.5% of TA was recovered. However, 19.6% of TA was recovered in the ceca.

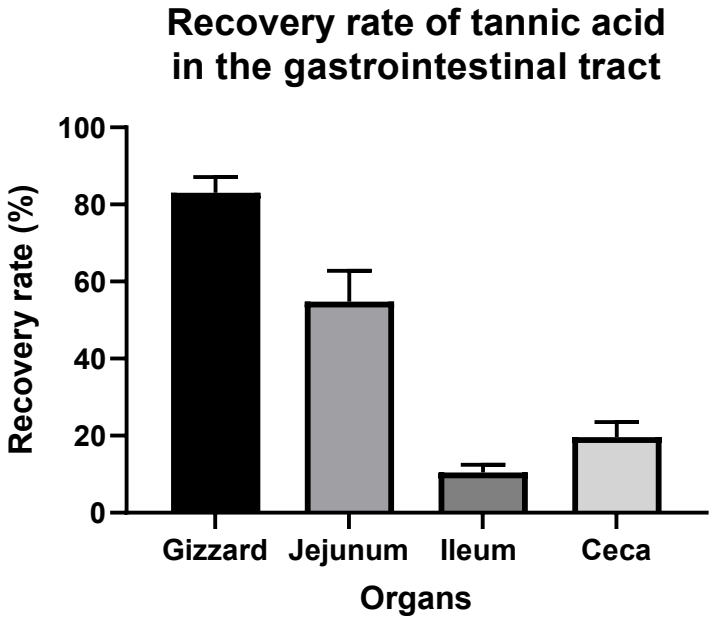


Figure 3.6. Recovery rate of tannic acid in the contents from the different part of the gastrointestinal tract in chickens D 35 fed 2,000 mg/kg tannic acid. Each value represents means ± SEM, $n = 4$.

3.4.6. Alpha and beta diversity of the ileal microbial communities

The alpha diversity indices of the ileal microbial communities including pielou evenness, faith phylogenetic diversity, observed features, and shannon entropy are presented in Table 3.2. On D 7, pielou evenness tended to be linearly decreased due to TA supplementation ($P = 0.091$), and observed features were quadratically increased due to TA supplementation ($P = 0.071$) in broilers infected with ST^{NR}.

Distance to each treatment according to the unweighted unifrac beta diversity analysis was shown in Figure 3.7. On D 21, *Salmonella* infected groups (CC, TA0.25, TA0.5, TA1, and TA2) had greater distance to the SCC group compared to the SCC group ($P < 0.01$). The TA1 group had great distance to the TA0.25 group compared to the TA0.25 group on D 7. The SCC and CC group had greater distance to the TA0.25 group compared to the TA0.25 group on D 21 ($P < 0.05$).

Table 3.2. Alpha diversity indices of the cecal microbial communities of broilers infected with *Salmonella* Typhimurium nalidixic acid resistant strain on D 7 and 21

Items	SCC[1]	ST^{NR}-challenged[2]						Polynomial contrast	
		CC	TA0.25	TA0.5	TA1	TA2	SEM	*P* value	Linear	Quadratic
D 7										
Pielou evenness	0.27	0.36	0.35	0.27	0.33	0.28	0.07	0.094	0.091	0.565
Faith phylogenetic diversity	7.53	6.72	6.06	6.86	7.94	6.77	1.52	0.259	0.464	0.151
Observed features	88	75.71	68.29	79	97.14	73.14	23.17	0.202	0.719	0.071
Shannon entropy	1.74	2.22	2.10	1.69	2.14	1.73	0.46	0.115	0.124	0.889
D 21										
Pielou evenness	0.34	0.34	0.38	0.37	0.34	0.39	0.09	0.687	0.509	0.665
Faith phylogenetic diversity	6.72	6.38	6.29	6.51	6.40	6.11	1.09	0.97	0.63	0.681
Observed features	80.86	83.86	84.43	85.29	83.00	86.00	7.96	0.997	0.847	0.871
Shannon entropy	2.19	2.18	2.44	2.38	2.16	2.52	0.55	0.674	0.478	0.66

SCC (sham-challenged control): broilers fed a basal diet and challenged with peptone water; CC (challenged control): broilers fed a basal diet and challenged with 0.5 mL of 1 × 10^8 CFU/mL of ST^{NR}; TA0.25 (tannic acid 0.25 g/kg): CC + 0.25 g/kg of tannic acid; TA0.5 (tannic acid 0.5 g/kg): CC + 0.5 g/kg of tannic acid; TA1 (tannic acid 1 g/kg): CC + 1 g/kg of tannic acid; and TA2 (tannic acid 2 mg/kg): CC + 2 g/kg of tannic acid;

[1] SCC vs CC (unpaired *t* test): * $0.05 < P < 0.10$, ** $P < 0.05$, *** $P < 0.01$

[2] ST^{NR} infected groups (CC, TA0.25, TA0.5, TA1, and TA2) were compared by PROC MIXED followed by the Tukey's multiple comparison test.

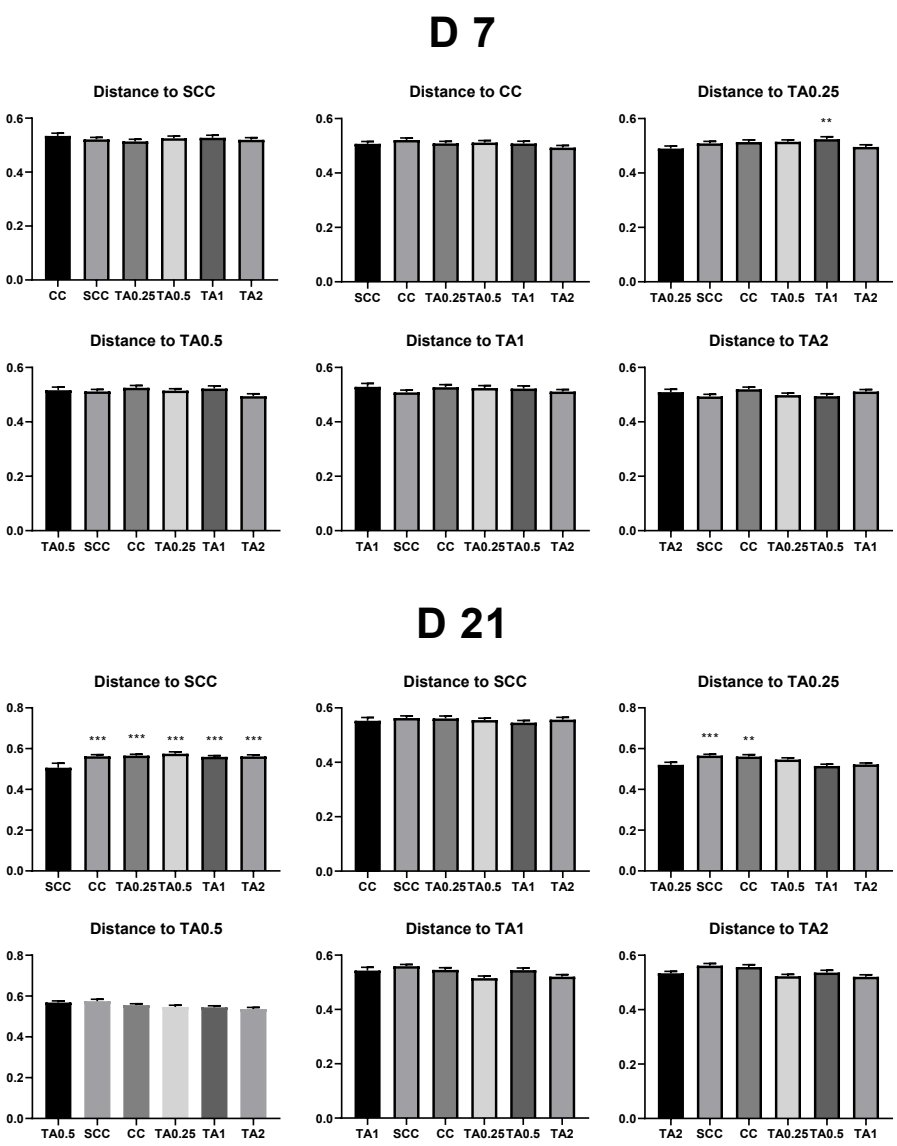


Figure 3.7. Beta diversity indice (unweighted unifrac) of the ileal microbial communities in the SCC ((sham-challenged control): broilers fed a control diet and administrated with phosphate buffered saline via oral gavage); CC ((challenged control): broilers fed a control diet and administrated with 1 mL 10^8 of *Salmonella* Typhimurium nalidixic acid resistant strain via oral gavage); TA0.25 ((tannic acid 0.25 g/kg): CC + 0.25 g/kg of tannic acid);

TA0.5 ((tannic acid 0.5 g/kg): CC + 0.5 g/kg of tannic acid); TA1 ((tannic acid 1 g/kg): CC + 1 g/kg of tannic acid); TA2 ((tannic acid 2 g/kg): CC + 2 g/kg of tannic acid) groups. Each treatment group was placed as the control group, and one-way ANOVA with Dunnett's post hoc test was used, and. ** denoted when $P < 0.05$, and *** denoted when $P < 0.01$. Each value represents means ± SEM, n = 7.

3.4.7. Phylum- and family- level composition of the cecal microbial communities

The phylum level composition of the ileal microbial communities was shown in Figure 3.7. However, no differences were observed in the phylum level among treatments on D 7 and 21 ($P > 0.1$).

Figure 3.8. and Figure 3.9. shows the phylum and family level compositions of the ileal microbial communities, respectively. On D 7, the T1 group had the highest relative abundance of the family Christensenellaceae, and TA supplementation quadratically increased the relative abundance of the family Christensenellaceae in broilers infected with ST^{NR} ($P < 0.05$). Infection of ST^{NR} tended to increase the relative abundance of the family Peptostreptococcaceae ($P = 0.087$), and TA supplementation linearly ($P < 0.05$) and quadratically (tendency; $P = 0.071$) decreased the relative abundance of the family Peptostreptococcaceae in broilers infected with ST^{NR}. On 21, the TA2 group had the highest relative abundance of the family Erysipelotrichaceae, and TA supplementation linearly ($P < 0.05$) and quadratically ($P = 0.06$) increase the relative abundance of the family Erysipelotrichaceae in broilers infected with ST^{NR}. The ST^{NR} infection increased the relative abundance of the family Lachnospiraceae.

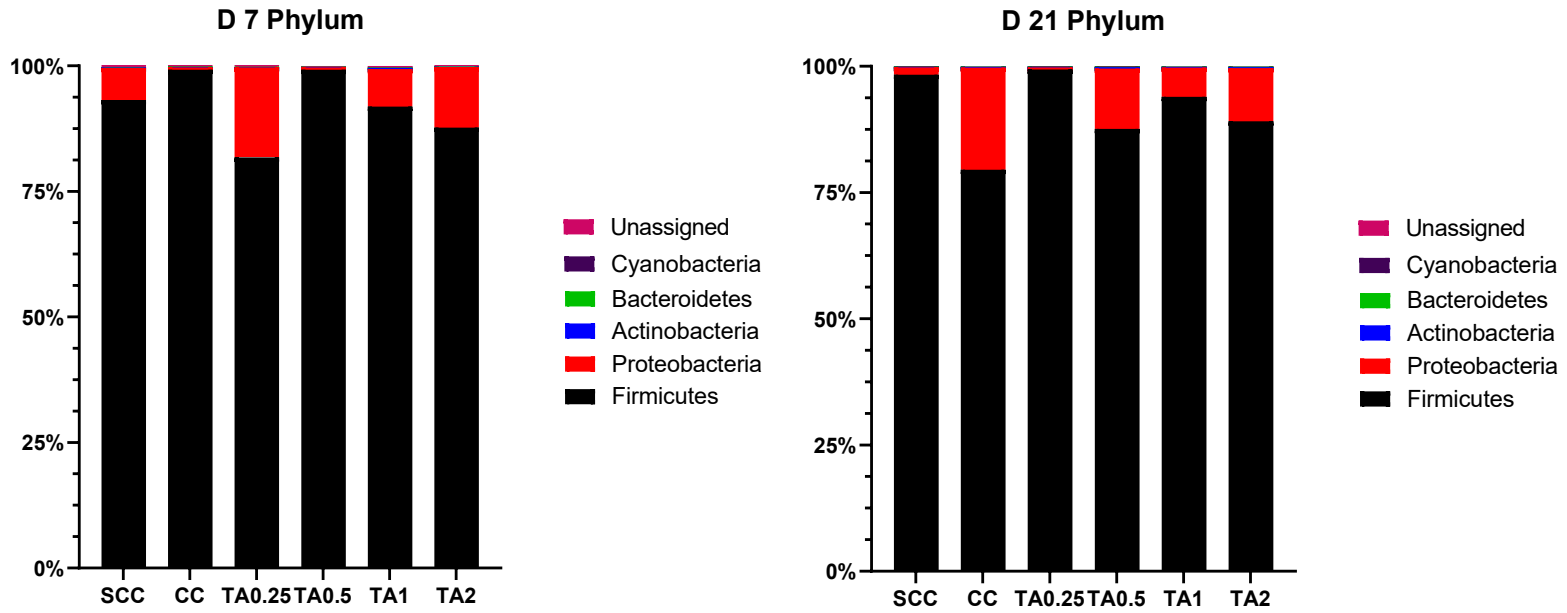


Figure 3.8. Phylum-level composition of the ileal microbial communities in the SCC ((sham-challenged control): broilers fed a control diet and administrated with phosphate buffered saline via oral gavage); CC ((challenged control): broilers fed a control diet and administrated with 1 mL 10^8 of *Salmonella* Typhimurium nalidixic acid resistant strain via oral gavage); TA0.25 ((tannic acid 0.25 g/kg): CC + 0.25 g/kg of tannic acid); TA0.5 ((tannic acid 0.5 g/kg): CC + 0.5 g/kg of tannic acid); TA1 ((tannic acid 1 g/kg): CC + 1 g/kg of tannic acid); TA2 ((tannic acid 2 g/kg): CC + 2 g/kg of tannic acid) groups on D 7 and 21. SCC was compared with CC (unpaired *t*-test). One-way ANOVA with Tukey's post hoc test was used. No statistical were observed at the phylum level ($P > 0.1$).

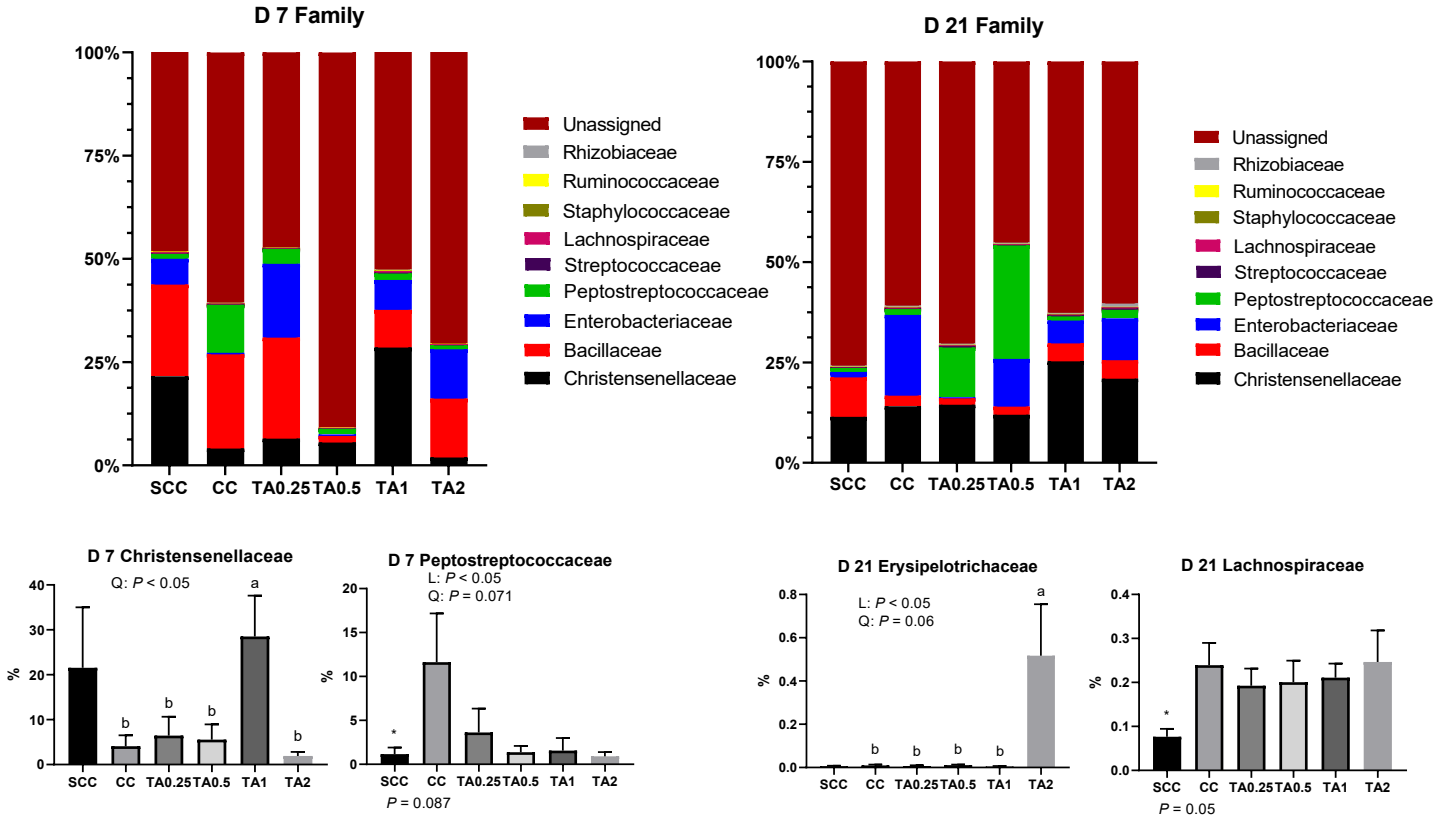


Figure 3.9. Family-level composition of the cecal microbial communities in the SCC ((sham-challenged control): broilers fed a control diet and administrated with phosphate buffered saline via oral gavage); CC ((challenged control): broilers fed a control diet and administrated with 1 mL 10^8 of *Salmonella* Typhimurium nalidixic acid resistant strain via oral gavage); TA0.25 ((tannic acid 0.25 g/kg):

CC + 0.25 g/kg of tannic acid); TA0.5 ((tannic acid 0.5 g/kg): CC + 0.5 g/kg of tannic acid); TA1 ((tannic acid 1 g/kg): CC + 1 g/kg of tannic acid); TA2 ((tannic acid 2 g/kg): CC + 2 g/kg of tannic acid) groups. SCC was compared with CC (unpaired *t*-test), and * denoted when the means were tended to be different ($0.05 \leq P \leq 0.10$). One-way ANOVA with Tukey's post hoc test was used, different letters indicated means were significantly different ($P < 0.05$).

3.5. DISCUSSION

The purpose of the study was to evaluate TA as an antibacterial agent against *Salmonella* spp. in *in vitro* and *in vivo* chicken conditions. The conditions to be natural antibacterial agents in chickens include 1) an antibacterial agent should have antibacterial effects against specific or general pathogens; 2) an antibacterial agent should not induce resistance in pathogens; 3) the antibacterial agents should be delivered to the target site where many most of the pathogens propagate (e.g., lower gastrointestinal tract); 4) an antibacterial agent should be heat stable under feed processing (e.g., pelleting) conditions; and 5) an antibacterial agent should be able to favorably modulate microbiota of chickens.

In the present study, TA was shown to have antibacterial effects against diverse *Salmonella enterica* serotypes including *S.* Typhimurium, *S.* Enteritidis, and *S.* Infantis using the well diffusion method. The TA are composed of a central core of glucose with 7 to 9 molecules of GA (Macáková et al., 2014). According to Kaczmarek (2020), proposed mode of actions of antibacterial effects of TA are 1) TA permeates bacterial cell wall up to the internal membrane and interferes the microbial metabolism; 2) TA deprives essential nutrients from microorganisms by chelating some proteins and iron (Chung et al., 1998); and 3) TA interacts with cell surface components including structure components and receptors, which increases permeability of bacterial cell wall and inhibits cell adhesion.

In this current study, *S.* Typhimurium 200 μg/mL nalidixic acid resistant strain (ST^{NR}) was selected among the *Salmonella enterica* serotypes for the further *in vitro* and *in vivo* antibacterial assays. Nalidixic acid is an antibiotic that has been commonly used in the poultry production, and *S.* Typhimurium NA resistant strain became prevalent to cause health issues

in chickens and humans (Campioni et al., 2017). There are already many antibiotic resistant strains of *Salmonella* spp. against tetracycline, ciprofloxacin, ampicillin, etc., and actions are required to control the spread and infection of antibiotic resistant strains of *Salmonella* spp. (Yuan and Guo, 2017). Furthermore, the NA resistant strain of *Salmonella* spp. have been frequently used as a research model due to their easy management and analyses (Adhikari et al., 2018). In addition, the higher resistant strains are preferred for easier isolation of the target strain. Therefore, ST^{NR} was determined as the model of the *S.* Typhimurium to further evaluate TA as an antibacterial agent in *in vitro* and *in vivo* chicken conditions.

To determine the MIC and sub-lethal concentrations, OD600 and visual turbidity was used. Visual turbidity measurement showed that lower than 40 μg/mL TA seemed initiated growth of ST^{NR}, and OD600 seemed to be increased lower than 40 μg/mL TA. However, OD600 was increased higher than 100 μg/mL TA potentially due to the formation of TA-nutrient complex in the broth. The MBC of TA against ST^{NR} determined was determined as 700 μg/mL by plating the culture on the agar plate. The TA can be considered as a bacteriostatic agent against ST^{NR}, which is defined as "an agent that inhibit the growth of bacteria while not necessarily killing them", because the MBC was 17.5 times higher than the MIC in the study. Higher than 4 of as a ratio of MBC to MIC can be considered as a bacteriostatic agent (Pankey and Sabath, 2004). Bacteriostatic agents potentially induce less antibacterial resistance than bactericidal agents (killing agents) because survived bacteria from bactericidal agents can be selected and spread and deliver resistance gene horizontally or vertically (Catry et al., 2003).

In the current study, GA did not form an inhibitory zone against diverse *Salmonella enterica* serotypes including *S.* Typhimurium, *S.* Enteritidis, and *S.* Infantis, and MIC of GA

against ST^{NR} of GA was 8,000 μg/mL, and MBC was above 10,000 μg/mL, which were much higher than the MIC and MBC of TA. Above 10,000 μg/mL of GA was not tried to determine the MBC because tannins higher than 10,000 μg/mL can show anti-nutritional effects in chickens and are not cost-effective as feed additives to be applied to the chickens (Choi and Kim, 2020). Consistently, Akiyama et al. (2001) reported that GA had limited antibacterial effects (MIC: 8,000 μg/mL) against diverse strains of *Staphylococcus aureus* compared to TA (MIC: 250 to 1,000 μg/mL). Limited antibacterial effects against *Salmonella enterica* serotypes of GA can be associated with limited protein precipitation capacity of GA in the study. Because GA are lack of protein precipitation ability (Zhao et al., 1997), the GA potentially may have a restricted ability to form complexes with bacterial components (e.g., lipopolysaccharides and bacterial enzymes). Therefore, the structure of TA to form complexes with nutrients is essential to exhibit antibacterial effects against *Salmonella enterica* serotypes.

Poultry diets are normally provided in a pelleted form, and the pelleting process includes conditioning, pelleting and cooling (Kiarie and Mills, 2019). The conditioning process is conducted by adding steam heat ranging 50°C to 90°C to amalgamate small particles of feed into large particles. During the conditioning process, pathogenic bacteria can be eliminated; however, feed additives can be deactivated and lose their potentials (Choi et al., 2020b). We hypothesized that simulated pellet temperature (50°C to 90°C) potentially decreases antimicrobial activities of TA because heat can hydrolyze TA to produce GA molecules (components of TA with glucose), which showed limited antimicrobial effects against ST^{NR} compared to the TA in the current study. In contrast, the present study showed that simulated pelleting temperature (60°C and 70°C) enhanced antimicrobial effects of TA against ST^{NR}

compared to the non-processed TA without affecting protein precipitation capacity. Antimicrobial effects of plant polyphenols are closely associated with their structure (Taguri et al., 2004). Potentially, simulated pelleting temperature at 60°C and 70°C modulated TA to have the optimal structure to form complexes with bacterial components by releasing some molecules of GA, which resulted in stronger antimicrobial effects against ST^{NR} without protein precipitation capacity of TA. Whereas antimicrobial effects were enhanced when TA were exposed to simulated pelleting temperature in this study, whether the actual pelleting process can improve antimicrobial effects of TA is still in question. This is because, conditioning time may not be enough to modulate structure of TA, and the actual pelleting process also provide wet steam and physical pressure with high temperature, which can induce TA to form complexes with dietary proteins or minerals, and this can potentially reduce antimicrobial effects of TA in chickens.

To test heat stability of TA in the harsher conditions, TA was autoclaved for different time in the current study. The autoclave process for 5, 10, 15, 20, 40 and 60 min did not alter antimicrobial effects against ST^{NR} and protein precipitation capacity of TA. In contrast, Kim et al. (2010) showed that autoclaved TA (dissolved in distilled water) had improved antimicrobial effects against some foodborne pathogens compared to the fresh TA. The differences could be due to the presence of solvent (pure TA v. TA in distilled water) or different strain of ST^{NR}. Whereas water containing TA can be heated up to 100°C with pressure in the autoclave conditions, autoclaved TA without any solvent in a tube possibly reached to more 200°C because Campbell Flake (2004) mentioned that when solid materials in a sealed vessel were autoclaved, the solid materials in the vessel can be exposed to more than 200°C

due to the accumulated pressure and continuous heat. Although pictures were not shown, TA melted when it was autoclaved more than 20 min in the current study, which implies that TA reached its boiling points (218°C). However, because antimicrobial effects and protein precipitation capacity of TA were maintained when TA was autoclaved for 60 min, we propose that the autoclave process would not fully hydrolyze TA into GA and glucose [(3) in Figure 2], and only tannase is known to fully hydrolyze TA into GA and glucose.(Mahmoud et al., 2018) Therefore, the heat process on TA with the appropriate consideration of temperature and time can enhance antimicrobial effects.

In addition to bacteriostatic effects of TA, anti-virulence effects (e.g., swarming and swimming motility and biofilm formation) of TA to restrict gut and internal organ colonization in chickens is also crucial (Yang et al., 2018). *Salmonella* spp. employ fimbriae or flagella to swim and swarm to move in the liquid environment (e.g., low agar concentration) and on semi-solid surfaces, respectively (Fraser and Hughes, 1999). Both swimming and swarming motility are vital for *Salmonella* spp. to invade the epithelial cells and immune cells, which results in colonization of *Salmonella* spp. in the intestine and internal organs (e.g., liver and spleen) of chickens. Furthermore, motility of *Salmonella* spp. is closely associated with biofilm formation because *Salmonella* spp. can produce biofilm once they are adhering to the intestinal wall (Deditius et al., 2015; Duan et al., 2013). *Salmonella* spp. in the biofilm, composed of polymeric substances that can protect colonies, are more resistant to antibacterials, physical stresses, and immune system in chickens (Peng, 2016). In the current study, sub-lethal concentrations of TA against ST^{NR} were determined by using OD600, and the determined concentrations (5, 10, and 20 μg/mL TA) inhibited swimming and swarming motility and

biofilm formation of ST^{NR}. The effects of TA on swimming and swarming motility and biofilm formation of other bacteria including *Pseudomonas aeruginosa* (O'May and Tufenkji, 2011), *Staphylococcus aureus* (Dong et al., 2018), and *Escherichia coli* (Hancock et al., 2010) were well known. The TA potentially interacted with bacterial flagellum, which may inhibit swimming and swarming motility and indirectly affect biofilm formation of ST^{NR} (O'May et al., 2012). In addition, bacterial motility inhibition and anti-biofilm effects of TA against ST^{NR} can be associated with the modulation of specific gene expression due to TA (Solano et al., 2002). Therefore, TA at sub-lethal concentrations can limit the virulence of ST^{NR} in addition to bacteriostatic activity.

Depending on the pH, binding property of TA with BSA (e.g., proteins) can be altered. According to Adamczyk et al. (2011), at pH near 4.7 (isoelectric point of BSA), almost 100% of TA were precipitated, and higher than pH 5 to 8, around 25% to less than 10% of TA were precipitated. According to Ravindran (2013), the pH of upper gastrointestinal tract of chickens can be 2.5 to 5 (e.g., proventriculus to duodenum), and at this pH range, TA would form complexes with endogenous and exogenous proteins in the gastrointestinal tracts. In the current study, the TA-BSA solution at simulated gastric pH (pH 3.75) had limited antibacterial effects compared to the pure TA without BSA. Potentially, TA-BSA complex limitedly interacts with components on the surface and in the inside of bacteria cells to exhibit antibacterial effects compared to the pure TA. If TA-protein complexes are not hydrolyzed in the lower intestine (pH 6 to 7), antibacterial effects of TA can be limited in the lower intestine, where most of *Salmonella* spp. propagate, in chickens. In the present study, pH 6.75 and 7.25 were determined as simulated lower intestinal pH of chickens (Ravindran, 2013). At pH 6.75, around 70% of

TA-BSA formed at gastric pH were hydrolyzed, and only at 30 and 60 min, antibacterial effects were restricted. However, at pH 7.25, most of TA-BSA (>90%) were hydrolyzed, and no differences were observed in the antibacterial effects at all time points. Consistently, Osawa and Walsh (1993) reported that TA-BSA complex was strongly dissociated at pH higher than 7. When TA-BSA were incubated at pH 7.25 for 120 min, their antibacterial seemed to be decreased while statistical differences were not obtained. This would be because in the high pH solution, TA can be hydrolyzed into GA (Osawa and Walsh, 1993), which have limited antibacterial effects compared to the TA. These data potentially suggest that TA has potential to exhibit antibacterial effects in the lower gastrointestinal tract (ileum and ceca) where most *Salmonella* spp. inhabit.

In addition to binding activity of TA with proteins, early absorption of TA in the gastrointestinal tract of chickens can limit antibacterial effects of TA in chickens. It is important for antibacterial agents to approach the target site to exhibit antibacterial effects (e.g., lower intestine for *Salmonella* spp.) in animals (Choi et al., 2020b). In the present study, the recovery rate of TA in the different parts (e.g., gizzard, jejunum, ileum, and ceca) of gastrointestinal tract was determined by analyzing concentration of titanium dioxide and TA in the feed and digesta. Approximately 54%, 10% and 20% of TA was recovered in the jejunum, ileum, and ceca, respectively, in the current study. In the current study, the recovery rate of TA in the ceca was higher than the ileum. This would be due to hydrolyzation of polysaccharides and polyphenols complex in the plant ingredients of the feed by cecal microbiome. Soybean meal, the main protein source in poultry diets, is known to contain low amount of TA, and polyphenols normally bind to polysaccharides and form polyphenols and polysaccharides

complex (Guo et al., 2022). The concentrations of total tannins in the ileum may be underestimated because the buffer solution (N, N-Dimethylformamide) cannot break down the polysaccharides to release tannins. However, ceca, which is the main area for microbial fermentation, potentially hydrolyzed polysaccharides and liberate tannins to interact with the ammonium ferric citrate solution. In the current study, around 80 to 90% TA were absorbed before approaching the lower intestine. A previous study by Carbonaro et al. (2001) reported that TA and TA-BSA were readily absorbable in the small intestine of rats. However, normally, around 500 to 5,000 mg/kg TA can be supplemented in poultry diets (Choi and Kim, 2020), hence approximately 50 to 500 mg/kg TA would be delivered to the lower intestine. Those concentrations are higher than the MIC of TA against ST^{NR}. To increase the recovery rate and antibacterial effects of TA in the lower gastrointestinal tract of chickens, encapsulation of TA should be considered if it is to be supplemented in poultry diets (Wang et al., 2020b).

Whereas colonization of a single strain pathogen in the gut can impair growth performance and gut health of chickens, it is more likely that the pathogen induces imbalance in the entire gut microbiota, which can negative affect growth performance and gut health of chickens (Borewicz et al., 2015). In addition to evaluation of antibacterial effects of TA against ST^{NR} in *in vitro* conditions, whether TA supplementation alters the ileal microbiome in chickens infected with ST^{NR} was investigated. In the current study, TA supplementation modulated alpha diversity indices such as Pielou's evenness and Observed features in the ileal microbial communities of chickens infected with ST^{NR}. TA supplementation linearly reduced evenness in the ileal microbial communities, which suggests that supplementation TA can selectively increase abundance of a few dominating taxa (Choi et al., 2015). Quadratically

increased observed features may indicate around 1,000 mg/kg TA can increase richness of ileal microbial communities in chickens infected with ST^{NR}. Higher microbial richness indicates higher microbiota matureness and stability in young chickens (Awad et al., 2016). In the present study, beta diversity analysis (unweighted unifrac) showed that ST^{NR} infection modulated the microbiome on D 21 rather than D 7. It was expected that the ileal microbiota would be altered on D 7 rather than D 21 because a higher number of ST^{NR} colonies were shown in culture-based counting on D 7. Potentially, colonization of ST^{NR} was not enough to alter microbiota on D 7 because a single strain accounts for limited part of the microbiome (Shang et al., 2018), but potentially, chronical effects of ST^{NR} changed the ileal microbiota in chickens on D 21. In the current study, TA supplementation modulated beta diversity of ileal microbial communities in chickens infected with ST^{NR}. Results of alpha and beta diversity analyses indicates that ST^{NR} infection and TA supplementation modulated ileal microbial communities of chickens.

Whereas it was expected that the relative abundance of the phylum Proteobacteria and the family Enterobacteriaceae on D 7 and 21 would be increased due to ST^{NR} infection because *Salmonella* spp. belong to the phylum Proteobacteria and the family Enterobacteriaceae, no differences were observed in the phylum Proteobacteria and the family Enterobacteriaceae on D 7 and 21. In contrast, in our companion study (Choi et al., 2022a), approximately log 6 and log 2 ST^{NR} per ceca g were observed on D 7 and 21, respectively, and on D 7, TA1 and TA2 groups had significantly lower ST^{NR} abundance compared to the CC group. The differences would be originated from different methods (culture-dependent v. culture-independent) or different organs (ceca v. ileum). Otherwise, ST^{NR} may have accounted for limited parts in the

entire ileal microbiome, which explains that different abundance of ST^{NR} could not lead to taxa differences in the ileal microbial communities. Our current study supports that culture-dependent and culture-independent methods should be accompanied to study microbiota of chickens (Gong and Yang, 2012).

In the present study, the TA1 group had the highest relative abundance of the family Christensenellaceae among ST^{NR} infected groups, and TA supplementation quadratically increased the relative abundance of the family Christensenellaceae. The family Christensenellaceae is known to have important role in protein and fiber fermentation in animals (Waters and Ley, 2019). In consistent, a previous study by reported that TA supplementation increased short chain fatty acids, microbial metabolites by fermenting fiber sources, in pigs (Koo and Nyachoti, 2019). In the present study, the relative abundance of the family Peptostreptococcaceae was increased due to ST^{NR} infection and linearly and quadratically decreased due to TA supplementation in chickens infected with ST^{NR} on D 7. The family Peptostreptococcaceae are known to closely positively correlated with pro-inflammatory cytokines and negatively correlated with gut barrier integrity of animals (Yan et al., 2019). Moreover, on D 21, TA supplementation increased the relative abundance of the family Erysipelotrichaceae, which is known to positively correlated to feed efficiency in chickens. Collectively, TA supplementation beneficially affected microbiota in broilers infected with ST^{NR}.

3.6. CONCLUSION

In conclusion, TA exhibited strong antibacterial effects against *Salmonella* Typhimurium in *in vitro* conditions, and considerate amount of TA can approach to lower

gastrointestinal tract. The TA supplementation positively modulated microbiota of chickens infected with ST^{NR}. Hence, TA has potential to be used as an antibacterial agent against *Salmonella* Typhimurium in broilers.

4.0. CHAPTER 4 MANUSCRIPT II

EFFECTS OF SUPPLEMENTAL TANNIC ACID ON GROWTH PERFORMANCE, GUT HEALTH, MICROBIOTA, AND FAT ACCUMULATION AND OPTIMAL CONCENTRATIONS OF TANNIC ACID IN BROILERS D 0 TO 21[1]

[1]**Choi, J.**, S. Yadav, J. Wang, B. J. Lorentz, J. M. Lourenco, T. R. Callaway, and W. K. Kim. Effects of supplemental tannic acid on growth performance, gut health, microbiota, and fat accumulation and optimal concentrations of tannic acid in broilers D 0 to 21. Front. physiol.:1452. Reprinted here with permission of the publisher.

4.1 ABSTRACT

This study was conducted to investigate the effects of different concentrations of tannic acid (TA) on growth performance, nutrient digestibility, gut health, immune system, oxidative status, microbial composition, volatile fatty acids (VFA), bone mineral density, and fat digestion and accumulation in broilers and to find optimal concentrations of TA for efficient growth and gut health in broilers. A total of 320 male Cobb500 broilers were randomly distributed to 4 treatments with 8 replicates including 1) tannic acid 0 (TA0): basal diet without TA; 2) tannic acid 0.5 (TA0.5): basal diet with 0.5 g/kg TA; 3) tannic acid 1.5 (TA1.5); and 4) tannic acid 2.5 (TA2.5). Supplemental TA at concentrations greater than 972 mg/kg tended to reduce BW on D 21 ($P = 0.057$). The TA2.5 had significantly lower apparent ileal digestibility (AID) of crude protein compared to the TA0 group. The AID of ether extract (EE) tended to be reduced by TA at concentrations greater than 525 mg/kg ($P = 0.08$). The jejunal lipase activities tended to be reduced by TA at concentrations less than 595.3 mg/kg ($P = 0.094$). TA linearly decreased goblet cell density in the crypts of the jejunum ($P < 0.05$) and reduced mRNA expression of mucin 2 at concentrations less than 784.9mg/kg and zonula occludens 2 at concentrations less than 892.6mg/kg ($P < 0.05$). The TA0.5 group had higher activities of liver superoxide dismutase compared to the TA0 group ($P < 0.05$). Bone mineral density tended to be linearly decreased by TA ($P = 0.05$), and the ratio of lean to fat was linearly decreased ($P < 0.05$). Total cecal VFA production tended to be linearly reduced by TA at concentrations greater than 850.9 mg/kg ($P = 0.073$). Supplemental TA tended to increase the relative abundance of the phylum Bacteroidetes ($P = 0.098$) and decrease the relative abundance of the phylum Proteobacteria ($P = 0.097$). The relative abundance of the family

Rikenellaceae was the lowest at 500mg/kg TA, and the relative abundance of the family Bacillaceae was the highest at 1,045mg/kg TA. Collectively, these results indicate that the optimum concentration of supplemental TA would range between 500 to 1,000 mg/kg; this range of TA supplementation can be used as an AGP alternative without negatively affecting growth performance in broilers under antibiotic-free conditions.

Keywords: tannic acid, gut health, apparent ileal digestibility, fat accumulation, microbiota, volatile fatty acids, chickens

4.1.INTRODUCTION

There are many challenges in poultry production such as pathogenic bacteria infection, parasitic infection, heat stress, etc. that can impair production efficiency (Choi and Kim, 2020). To cope with these issues, antibiotics as growth promoters (AGP) have been supplemented in poultry diets (Pedroso et al., 2006). However, the continuous use of AGP has led to an increase in the spread of resistant bacteria and their resistant genes (Zhang et al., 2015). Due to the public demand to reduce the use of AGP, many countries have banned or restricted the use of AGP in poultry (Luise et al., 2022). Many bioactive compounds including essential oils (Yang et al., 2021), plant extracts (Mogire et al., 2021; Yadav et al., 2022a), organic acids (Pham et al., 2020), exogenous enzymes (Lu et al., 2020a), probiotics (Adhikari and Kim, 2017), and prebiotics (Teng and Kim, 2018) were studied to replace AGP in poultry production. Potential AGP replacements should be cost-effective and be able to improve growth performance via exhibiting antimicrobial, antioxidative, and anti-inflammatory effects in chickens (Yang et al., 2015a).

Tannins, plant secondary metabolites, are polyphenol compounds that can precipitate proteins (Sarni-Manchado et al., 1999). Tannins can be mainly categorized into condensed tannins and hydrolysable tannins (Huang et al., 2018). Hydrolysable tannins may have higher bioavailability compared to condensed tannins because hydrolysable tannins can be hydrolyzed and absorbed in the gastrointestinal tract of chickens (Choi and Kim, 2020). Tannic acid (TA), a standard of hydrolysable tannins, is considered as a potential AGP alternative mainly due to their strong antimicrobial, antioxidative, and anti-inflammatory effects, while the high concentrations of TA can exhibit anti-nutritional effects in chickens (Choi and Kim,

2020). TA supplementation reduced oocyst shedding of *E. maxima* and improved nutrient utilization and gut barrier integrity in broilers infected with *E. maxima* (Choi et al., 2022b). A previous study by Ebrahim et al. (2015) also reported that supplemental TA was beneficial to enhance the fatty acid profile of breast muscle of broilers subjected to heat stress. Along with the investigation of beneficial effects of supplemental TA in broilers under challenging conditions, the effects of supplemental TA on growth performance, nutrient digestibility, and gut health in broilers without any challenging conditions should be investigated to apply supplemental TA in poultry production. It is important to identify the optimal concentration range of supplemental TA without anti-nutritional effects in broilers under healthy conditions because TA can be beneficial as well as detrimental depending on its supplemental concentration. Moreover, supplemental TA potentially can ameliorate oxidative stress and inflammation caused by antinutritional factors in soybean meal and fast growth rate in modern broilers (Cheng et al., 2019; Choi et al., 2020a). Therefore, the hypothesis of the study was that supplemental TA at optimal concentrations would enhance growth performance, nutrient digestibility, gut health, immune system, oxidative status, microbiota, and body composition in broilers. The objectives of the study were 1) to investigate the effects of different concentrations of supplemental TA on growth performance, nutrient digestibility, gut health, immune system, oxidative status, microbial composition and activities, bone mineral density, and fat digestion and accumulation in broilers; and 2) to find optimal concentrations of TA for efficient growth and gut health in broilers reared D 0 to 21.

4.2. MATERIALS AND METHODS

4.2.1. Experimental design, diets, management, and growth performance

The current study was approved by the Institutional Animal Care and Use Committee at the University of Georgia, Athens, GA. A total of 320 one-day old male Cobb500 broilers were randomly allocated to 4 treatments with 8 replicates per treatment of 10 birds per pen in a completely randomized design. The basal diets for the starter (D 0 to 14) and grower (D 14 to 21) phase were formulated to meet or exceed energy and nutrient requirements based on Cobb Broiler Management Guide (Cobb 2018) as shown in Table 4.1. Four dietary treatments included 1) basal diet without TA (ACS reagent; Sigma–Aldrich, St Louis, MO; TA0); 2) basal diet with 0.5 g/kg TA (TA0.5); 3) basal diet with 1.5 g/kg TA (TA1.5); and 4) basal diet with 2.5 g/kg TA (TA2.5). During the whole experimental period, birds were allowed to eat and drink freely (D 0 to 21), and temperature and light were controlled in accordance with Cobb Broiler Management Guide (Cobb 2018). On D 7, 14, and 21, body weight (BW) and feed disappearance were recorded to calculate average daily gain (ADG), average daily feed intake (ADFI), and feed conversion ratio (FCR).

Table 4.1. Ingredients and nutrient compositions of basal diets (As-fed basis)

Items	D 0 to 14	D 14 to 21
Ingredients		
Corn	650.97	683.74
Soybean meal (480 g crude protein/kg)	295.05	260.05
Defluorinated Phosphate	16.62	14.96
Filler[1]	10	10
Soybean oil	8.27	12.91
Limestone	6.45	6.24
DL-Methionine 99%	3.18	2.94
L-Lysine HCl 78%	3.01	2.85
Vitamin Premix[2]	2.5	2.5
Sodium chloride	1.45	1.66
L-threonine	1.20	0.86
Mineral Premix[3]	0.8	0.8
Monensin sodium[4]	0.5	0.5
Total	1,000	1,000
Calculated energy and nutrient value, %		
Metabolizable energy, Kcal/kg	3,000	3,060
Crude protein	20.59	19.13
SID[5] Methionine	0.61	0.57
SID Total sulfur amino acids	0.88	0.82
SID Lysine	1.17	1.07
SID Threonine	0.78	0.7
Total calcium	0.87	0.8
Available phosphate	0.44	0.4

[1] Sand and tannic acid were added to obtain desired tannic acid concentrations in the feed as follows: Tannic acid 0 (TA0): sand 10 g/kg + tannic acid 0 g/kg; Tannic acid 0.5 (TA0.5): sand 9.5 g/kg + tannic acid 0.5 g/kg; Tannic acid 1.5 (TA1.5): sand 8.5 g/kg + tannic acid 1.5 g/kg; Tannic acid 2.5 (TA2.5): sand 7.5 g/kg + tannic acid 2.5 g/kg; Tannic acid was purchased from Sigma–Aldrich (St Louis, MO).

[2] Vitamin mix provided the following in mg/100 g diet: thiamine-HCl, 1.5; riboflavin, 1.5; nicotinic acid amide, 15; folic acid, 7.5; pyridoxine-HCl, 1.2; D-biotin 3, 1.5; vitamin B-12 (source concentrations, 0.1%), 2; choline-Cl, 3; D-calcium pantothenate, 4; menadione sodium bisulfite, 1.98; α-tocopherol acetate (source 500,000 IU/g), 22.8; cholecalciferol (source 5,000,000 IU/g), 0.09; retinyl palmitate (source 500,000 IU/g), 2.8; ethoxyquin, 13.34; I-inositol, 2.5; and dextrose, 762.2

[3] Mineral mix provided the following in g/100 g diet: $Ca(H_2PO_4)_2 \cdot H_2O$, 3.62; $CaCO_3$, 1.48; KH_2PO_4, 1.00; Na_2SeO_4, 0.0002; $MnSO_4 \cdot H_2O$, 0.035; $FeSO_4 \cdot 7H_2O$, 0.05; $MgSO_4 \cdot 7H_2O$, 0.62; KIO_3, 0.001; NaCl, 0.60; $CuSO_4 \cdot 5H_2O$, 0.008; $ZnCO_3$, 0.015; $CoCl_2 \cdot 6H_2O$, 0.00032; $NaMoO_4 \cdot 2H_2O$, 0.0011; KCl, 0.10; and dextrose, 0.40.

[4] Coban 90, Elanco Animal Health, Greenfield, IN

5 SID: standardized ileal digestible

4.2.2. Sample collection

On D 21, birds were euthanized via cervical dislocation. Afterwards, blood samples were collected in heparin free vacutainers (Grainer Bio-One, Kremsmuenster, Austria) from the hearts. After being allowed to clot for 1 h at room temperature, the samples were centrifuge at 1,000 × *g* for 10 min to recover serum. the serum samples were stored at – 80°C for further analyses. The ceca and liver samples and a 10 cm segment of the mid-jejunum samples were collected and snap-frozen using liquid nitrogen and stored at – 80°C for future analyses. A 2 cm segment of the mid-jejunum and mid-ceca were collected and fixed in a 10% formaldehyde solution. All tissue samples except ceca samples were washed with PBS to remove blood and digesta. From 10 cm below Meckel's diverticulum to the upper 10 cm of the ileo-cecal-colic junction, ileal digesta samples were collected.

4.2.3. Apparent ileal digestibility of dry matter, organic matter, ash, crude protein, and ether extract

According to Short et al. (1996), the concentrations of titanium dioxide were measured in oven-dried samples (0.3 g for ileal digesta and 0.5 g for the feed samples). The crude protein (CP) was determined by nitrogen combustion analyses according to AOAC International (2000) analytical method 990.03. The ether extract (EE) content was analyzed according to AOAC International (2000) analytical method 942.05. Apparent ileal digestibility (AID) of dry matter (DM), organic matter (OM), ash, CP, and EE were calculated according to Lin and Olukosi (2021).

4.2.4. Jejunal and cecal morphology and goblet cell counting

The alcian blue/ period acid-Schiff (AB/PAS) staining was performed to measure villus height (VH), crypts depth (CD), and the VH:CD ratio and to count the number of goblet cells in VH and CD in the jejunum and ceca (only crypts) samples. Samples were fixed in 10% neutral-buffered formalin for further analyses. Afterwards, the jejunal and cecal samples were cut cross-sectionally to 0.5 cm and put in cassettes. The sections were stained with alcian blue for 15 min and washed with distilled water. The samples were treated with periodic acid for 5 min and washed with distilled water. Subsequently, the samples were stained with Schiff's reagents for 10 min and washed with distilled water. Finally, the samples were counterstained in haematoxylin for 1 min and washed and dehydrated. The stained sections were pictured with a microscope (BZ-X810; Keyence, Osaka, Japan). Pictured images (4×) were analyzed to measure villus height (VH) and crypt depth (CD) using ImageJ (National Institutes of Health, Bethesda, MD).

4.2.5. Activities of brush border digestive enzymes and serum alkaline phosphatase in the mid-jejunum

Approximately 100 mg of the whole mid-jejunum samples with 2 mL PBS were homogenized using a beads beater (Biospec Products, Bartlesville, OK). The supernatants of homogenized samples after the centrifugation at 4°C and 12,000 × *g* for 15 min were collected to analyze their protein contents using Pierce BCA Protein Assay Kits (Thermo Fisher Scientific, MA) with a 10-time sample dilution. By using the supernatants, activities of maltase and sucrase were determined according to Fan et al. (2004). Activities of alanine-aminopeptidase (APN) were analyzed according to Maroux et al. (1973). Activities of serum

alkaline phosphatase and intestinal alkaline phosphatase (IAP) were determined according to the method from Lackeyram et al. (2010). Lipase activities were determined according to Elgharbawy et al. (2018). The activities of digestive enzymes were expressed as values per mg protein.

4.2.6. RNA extraction and real-time reverse transcription (RT)-PCR analysis

Approximately 100 mg of the whole mid-jejunum and liver samples were homogenized in QIAzol lysis reagents (Qiagen, Valencia, CA), and RNAs were extracted according to the manufacturer's procedure. A NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific) was used to determine RNA quantity and purity. One microgram of RNA was utilized to make the first-strand cDNA using high-capacity cDNA synthesis kits (Applied Biosystems, Foster City, CA). The primers used in the study were presented in Table 4.2. Real-time PCR was performed using SYBR Green Master Mix with a Step One thermocycler (Applied Biosystem). The final PCR volume (10 μL) contained 5 μL of SYBR Green Master Mix (Applied Biosystems), 1.5 μL of cDNA, 0.5 μL of forward and reverse primers (10 μM), and 2.5 μL of water. The thermal cycle condition for all reactions included 95°C denature for 10 min, 40 cycles at 95°C for 15 sec and 60°C for 1 min, 95°C for 15 sec, 60°C for 1 min, and 95°C for 15 sec. Several PCR products mixed with DNA gel loading dye (6×; Thermo Fisher Scientific) from each gene were electrophoresed on a 3% agarose gel in Tris-acetate-EDTA buffer and visualized by 0.01% ethidium bromide, and a melting curve of each gene was checked to confirm the specificity of each PCR product. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and beta actin were used as housekeeping genes (reference genes). The target mRNA abundance was normalized with geometric means of housekeeping genes

(Vandesompele et al., 2002). Relative mRNA abundance was determined by using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The negative control, containing no cDNA, was included in each run, and each sample was run in duplicate.

Table 4.2. Primers used in the study[1]

Genes	Sequence, 5′ to 3′	Amplicon
GAPDH	F: GCT AAG GCT GTG GGG AAA GT R: TCA GCA GCA GCC TTC ACT AC	161
Beta actin	F: CAA CAC AGT GCT GTC TGG TGG TA R: ATC GTA CTC CTG CTT GCT GAT CC	205
B0AT1	F: GGG TTT TGT GTT GGC TTA GGA A R: TCC ATG GCT CTG GCA GAG AT	60
EAAT3	F: TGC TGC TTT GGA TTC CAG TGT R: AGC AAT GAC TGT AGT GCA GAA GTA ATA TAT G	79
SGLT1	F: GCC ATG GCC AGG GCT TA R: CAA TAA CCT GAT CTG TGC ACC AGT A	71
PEPT1	F: CCC CTG AGG AGG ATC CTT R: CAA AAG AGC AGC AAC GA	66
MUC2	F: ATG CGA TGT TAA CAC AGG ACT C R: GTG GAG CAC AGC AGA CTT TG	110
JAM2	F: AGC CTC AAA TGG GAT TGG ATT R: CAT CAA CTT GCA TTC GCT TCA	59
ZO2	F: ATC CAA GAA GGC ACC TCA GC R: CAT CCT CCC GAA CAA TGC	100
IL1β	F: TGC CTG CAG AAG AAG CCT CG R: GAC GGG CTC AAA AAC CTC CT	204
IL2	F: TTG GCT GTA TTT CGG TAG CA R: GTG CAC TCC TGG GTC TCA GT	59
IL6	F: ATA AAT CCC GAT GAA GTG G R: CTC ACG GTC TTC TCC ATA AA	146
NFκB	F: GAA GGA ATC GTA CCG GGA ACA R: CTC AGA GGG CCT TGT GAC AGT AA	131
ACACA	F: ACTGAATTGGTGCTGGATGA R: GGGTCTTGAGGGTCATTTTC	103
ACOX1	F: ATGGAATTGCAGACCCAGA R: GAGAAGGGTAGGGAGGAACA	107
APOB	F: CAAATGCCATGTCCAAACA R: TCCTCTCTTGAGATTGAGGACA	110
CD36	F: CTGGGAAGGTTACTGCGATT R: GGATCTGCAAATGTCAGAGG	109
CPT1A	F: CAGATGTTATGACAGGTGGTTTG R: CCCACAGGTGTCCAACAATA	119
FABP2	F: CTCTTGGAACCTGGAAGGAA R: CTCCTTCGTACACGTAGGTCTG	125
FABP4	F: CATAACCCTAGACAATGGCACA R: ATTCCACCAGCAGGTTCC	101

FASN	F: GGCAACTATCCTTCCCAAAA R: GAGGGAGATCTTCCCACTCA	118
GCG	F: AAGAAATGGCCAACAAGGAC R: GCCTTCAGCATGTCTCTCAA	107
PPARγ	F: GATGACAGTGACCTGGCAAT R: TCCAAAGCTTGCAACAGATT	116

[1]GAPDH, glyceraldehyde 3-phosphate dehydrogenase; B0AT1, sodium-dependent neutral amino acid transporter 1; EAAT3, excitatory amino acid transporter 3; SGLT1, sodium glucose transporter 1; PepT1, peptide transporter 1; MUC2, mucin 2; JAM2, Junctional adhesion molecule 2; Zonula occludens 2; zonula occludens 2; IL, interleukin; NFκB, nuclear factor kappa-light-chain-enhancer of activated B cells; ACACA, acetyl-CoA carboxylase alpha; ACOX1, acyl-CoA oxidase 1; APOB, apolipoportine B; CD36, clusters of differentiation 36; CPT1A, carnitine palmitoyltransferase 1A; FABP, fatty acid binding protein; FASN, fatty acid synthase; GCG, glucacon; PPARγ, peroxisome proliferator-activated receptor gamma

4.2.7. Total antioxidant capacity, glutathione, oxidized GSH, and activities of superoxide dismutase in the liver

Approximately 100 mg of the liver samples were homogenized with 1 mL of the designated solution for each analysis including total antioxidant capacity (TAC), glutathione (GSH) and oxidized GSH (GSSG), and superoxide dismutase (SOD) using a beads beater (Biospec Products, Bartlesville, OK). The TAC of the liver tissues were determined using colorimetric microplate assay kits for Total antioxidant capacity (TA02, Oxford Biomedical Research, Oxford, MI) according to Choi et al. (2020c). The GSH and GSSG in the liver were analyzed by using Caymans GSH assay kits (Cayman Chemical, Ann Arbor, MI) with a 20-time sample dilution. The activities of SOD in the liver were analyzed using Caymans SOD assay kit (Cayman Chemical) with a 400-time sample dilution. The supernatants of homogenized samples after the centrifugation at 4°C and 12,000 × *g* for 15 min were collected to analyze their protein content using Pierce BCA protein assay kits (Thermo Fisher Scientific) with a 20-time sample dilution. The TAC, GSH and GSSG concentrations, and SOD activities were expressed as values per mg protein.

4.2.8. Body composition analyses by using dual-energy X-ray absorptiometry

On D 21, three birds per pen were euthanized by cervical dislocation. According to the method of Wang et al. (2021b), dual-energy X-ray absorptiometry (DEXA, GE Healthcare, Madison, WI) was used to measure bone mineral density (g/cm2), bone mineral content (g), tissue weight (g), fat weight (g), lean weight (g), body fat percentage (%), and lean:fat (g/g)

4.2.9. Cecal volatile fatty acid analysis

Concentrations of volatile fatty acids (VFA) in cecal digesta were determined according to the method of Choi et al. (2021). The samples were snap-frozen in liquid nitrogen and stored at −80°C for further analyses. Once thawed, 0.5 g samples were diluted with 3 mL distilled water. The samples were vigorously homogenized for 1 min and subsequently frozen at −20°C. After the samples were thawed, the samples were centrifuged at 10,000 × *g* for 10 min, and 850 μL of the supernatants were collected, mixed with 170 μL of the fresh 25% (wt/vol) meta-phosphoric acid solution, and immediately frozen at − 20°C overnight. The samples were centrifuged at 10,000 × *g* for 10 min, and 800 μL of the supernatant was collected and mixed with 1,600 μL ethyl acetate. The samples were vigorously homogenized for 10 sec and allowed to settle for 5 min. The top layer was transferred to a screw-thread vial and analyzed in a gas chromatograph (Shimadzu GC-2010 plus; Shimadzu Corporation, Kyoto, Japan) equipped with an autoinjector (AOC-20i; Shimadzu Corporation, Kyoto, Japan). A capillary column (Zebron ZB-FFAP; 30 m × 0.32 mm × 0.25 μm; Phenomenex Inc., Torrance, CA) was utilized for the separation of the VFA. The sample injection volume was set at 1 mL, and helium was used as the carrier gas. The column temperature was initially set at 110°C and gradually increased to 200°C over the course of 6 min. The flame ionization detector was set at 350°C.

4.2.10. DNA extraction and microbiome analysis in the cecal content

DNA was extracted from the contents of mid-ceca by using QIAamp® DNA stool mini kits (Qiagen GmbH, Hilden, Germany) according to manufacturer procedure. Quality and quantity of extracted DNA was checked using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). The 16s rRNA gene sequencing was conducted by LC sciences, LLC

(Houston, TX). The V3 and V4 regions were amplified using 338F (5-CCTACGGGNGGCWGCAG-3)/806R (5-GACTACHVGGGTATCTAATCC-3) primers by PCR procedures as follows. The 5' ends of the primers were tagged with specific barcodes per sample and sequencing universal primers. The PCR amplification was conducted in a total volume of 25 μL reaction mixture containing 25 ng of template DNA, 12.5 μL PCR Premix, 2.5 μL of each primer, and PCR-grade water to adjust the volume. The PCR conditions to amplify the prokaryotic 16S fragments consisted of an initial denaturation at 98℃ for 30 sec; 32 cycles of denaturation at 98℃ for 10 sec, annealing at 54℃ for 30 sec, and extension at 72℃ for 45 sec; and then final extension at 72 ℃ for 10 minutes. The PCR products were checked with 2% agarose gel electrophoresis. The PCR products were purified using AMPure XT beads (Beckman Coulter Genomics, Danvers, MA) and quantified by Qubit (Invitrogen, Carlsbad, CA). The amplicon pools were prepared for sequencing, and the size and quantity of the amplicon library were assessed on Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA) and with the library quantification kit for Illumina (Kapa Biosciences, Woburn, MA), respectively. The libraries were sequenced on NovaSeq PE250 platform. For 16s rRNA analyses, QIIME2 (version 2021.11) was used (Bolyen et al., 2019). By using the QIIME2 demux emp-paired function and QIIME2 plugin DADA2, sequences were demultiplexed and denoised, respectively. The lowest frequency was 14,500, and the highest frequency was 32,278 after trimming the sequences of all samples. GreenGenes database (version 13.8) at the 99% operational taxonomic units (OTUs) for the region (515F/806R) was used for taxonomical classification. Phylum- and family-level compositions were presented. Alpha diversity and

beta diversity were analyzed by QIIME2's built in functions. Heatmap of the top 30 most abundant features was generated by QIIME2's built in functions.

4.2.11. Statistical analyses

SAS (version 9.4; SAS Inst. Inc., Cary, NC) and GraphPad Prism (Version 9.1.0; GraphPad Software, San Diego, CA) were utilized for statistical analyses and graph construction. Treatment groups were compared using one-way ANOVA in a completely randomized design followed by the Tukey's comparison test. Orthogonal polynomial contrasts were used to evaluate the significance of linear or quadratic effects of supplemental TA in broilers. To calculate the optimal concentration of TA in broilers, the segmented linear regression analysis was conducted, and inflection point (*X0)* with *P* values for slope 1 (< *X0*) and slope 2 (> *X0*) were presented. Significance level was set at $P < 0.05$, and tendencies were also determined at $0.05 < P \leq 0.10$ (Choi et al., 2021). Correlation between the cecal microbial composition with VFA and growth performance, fat accumulation, bone health, fat metabolism mRNA expression, immune system, gut barrier integrity, brush border digestive enzymes, antioxidant capacity, AID, nutrient transporters, and VFA parameters were analyzed, parameters with significant differences ($P < 0.05$) were presented.

4.3. RESULTS

4.3.1. Growth performance

The results of growth performance in broilers fed different concentrations of TA are presented in Figure 4.1. On D 7, the TA2.5 group had lower BW compared to the TA0 group ($P < 0.05$), and supplemental TA linearly ($P < 0.05$) and quadratically (tendency; $P = 0.095$) modulated BW. On D 14, BW and ADFI were linearly reduced by supplemental TA ($P < 0.05$).

On D 21, supplemental TA tended to reduce BW (P = 0.082), and at concentrations greater than 972 mg/kg supplemental TA tended to reduce BW in broilers (P = 0.057).

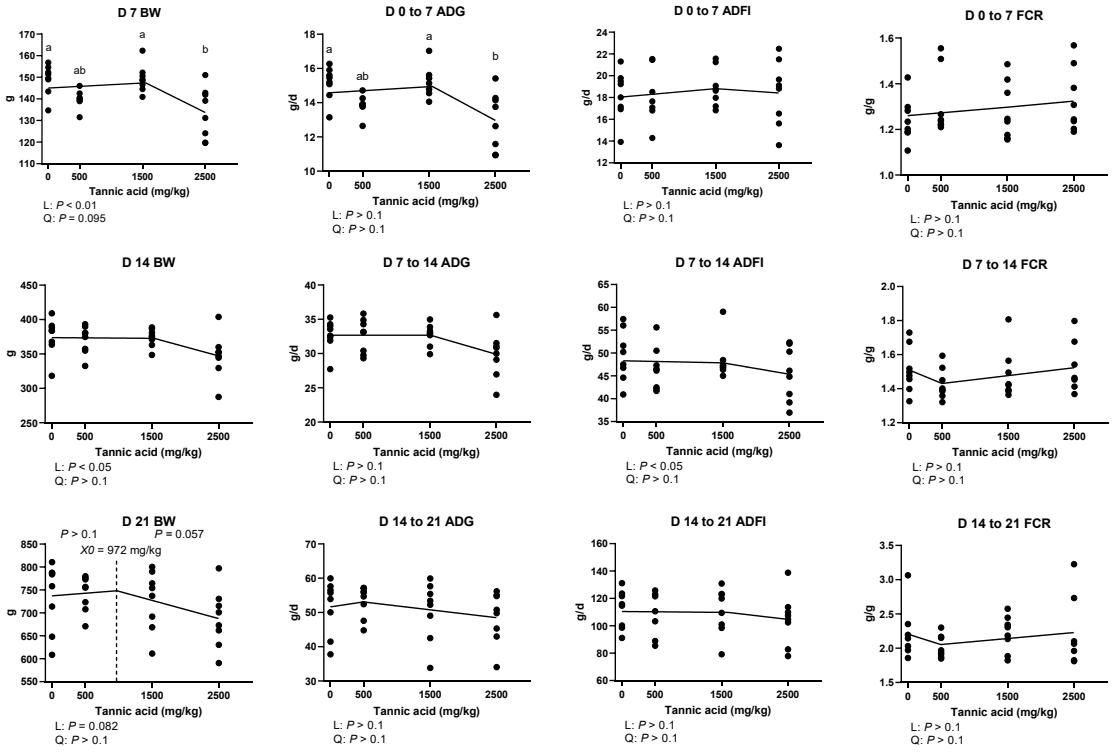


Figure 4.1. Growth performance parameters (BW, ADG, ADFI, and FCR) of broiler chickens fed different concentrations (0, 0.5, 1.5, and 2.5 g/kg) of tannic acid on D 7, 14, and 21. The treatment groups were compared using one-way ANOVA followed by the Tukey's multiple comparison. Different superscripts differ at $P < 0.05$. Orthogonal polynomial contrasts analysis was conducted to see linear pattern (L) and quadratic pattern (Q) among the treatments. To calculate the optimal concentration of tannic acid in broilers, segmented linear regression was conducted, and *X0* (inflection point) with *P* values for slope 1 (lower than *X0*) and slope 2 (higher than *X0*) were presented.

4.3.2. Apparent ileal digestibility of nutrients

The results of AID of DM, OM, ash, CP, and EE in broilers fed different concentrations of TA are shown in Figure 4.2. The AID of DM, OM, and ash was not affected by supplemental TA (Figure 4.2.; $P > 0.1$). However, AID of CP was significantly reduced by supplemental TA ($P < 0.01$). The AID of EE tended to be reduced by TA at concentrations less than 525 mg/kg ($P = 0.08$).

4.3.3. Jejunal and cecal morphology and goblet cell density

Jejunal and cecal morphology and goblet cell density in the broilers fed different concentrations of TA are presented in Figure 4.3. No differences were observed in the jejunal VH and CD and cecal CD ($P > 0.1$). However, jejunal goblet cell number per 100 μm CD was significantly reduced by supplemental TA ($P < 0.05$).

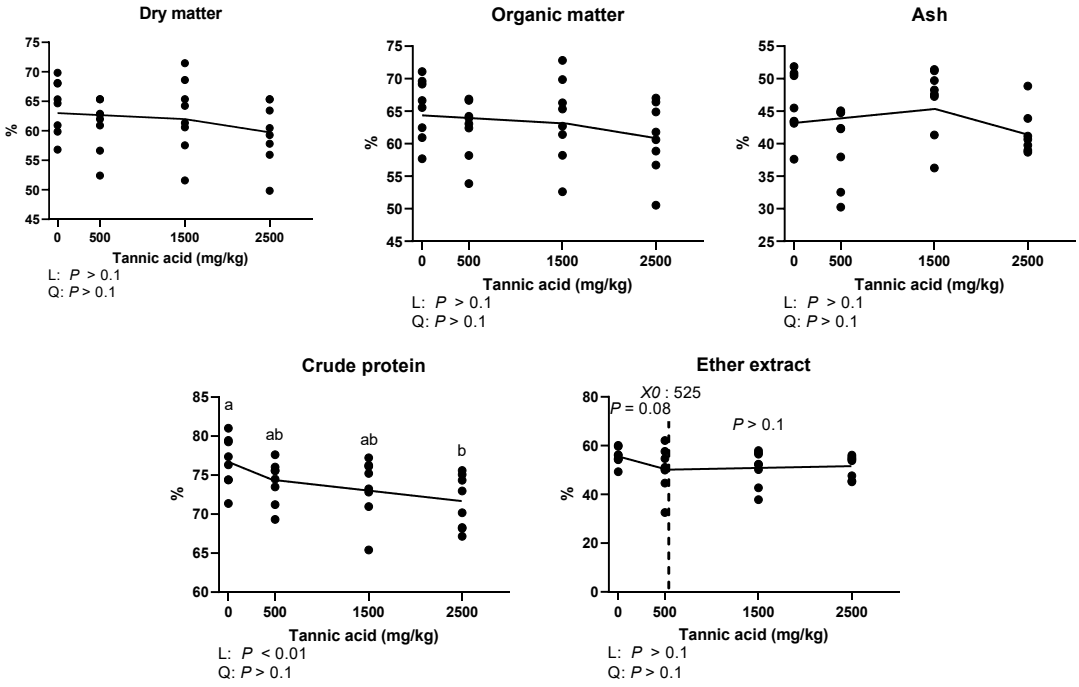


Figure 4.2. Apparent ileal digestibility of dry matter, organic matter, ash, crude protein, ether extract in broiler chickens fed different concentrations (0, 0.5, 1.5, and 2.5 g/kg) of tannic acid on D 21. The treatment groups were compared using one-way ANOVA followed by the Tukey's multiple comparison. Different superscripts differ at $P < 0.05$. Orthogonal polynomial contrasts analysis was conducted to see linear pattern (L) and quadratic pattern (Q) among the treatments. To calculate the optimal concentration of tannic acid in broilers, segmented linear regression was conducted, and *X0* (inflection point) with *P* values for slope 1 (lower than *X0*) and slope 2 (higher than *X0*) were presented.

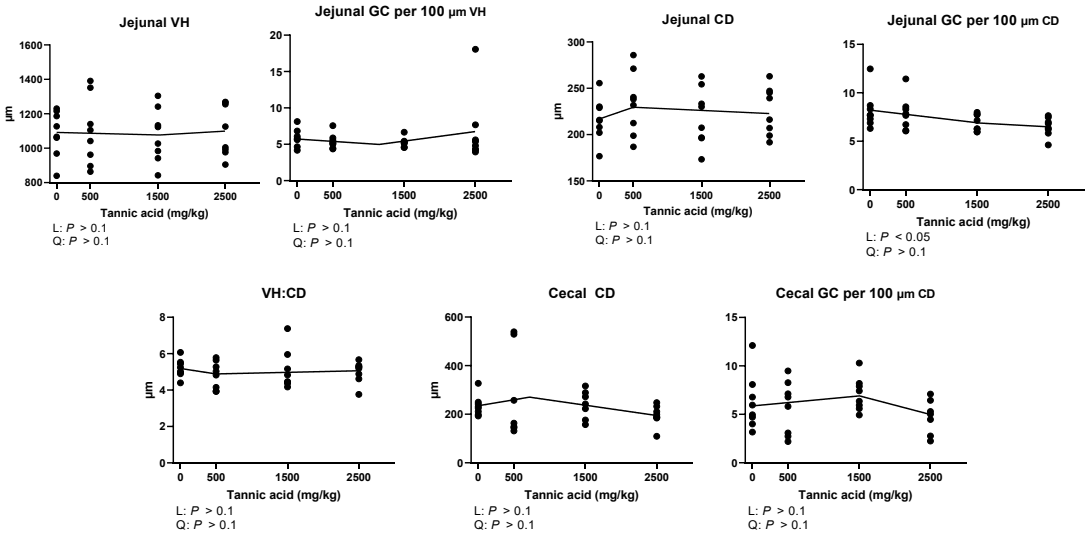


Figure 4.3. Jejunal morphology [villus height (VH), crypts depth (CD), and VH:CD] and goblet cell (GC) counting per 100 μm VH and CD and cecal CD and goblet cell counting per 100 μm CD in broiler chickens fed different concentrations (0, 0.5, 1.5, and 2.5 g/kg) of tannic acid on D 21. The treatment groups were compared using one-way ANOVA followed by the Tukey's multiple comparison. Different superscripts differ at $P < 0.05$. Orthogonal polynomial contrasts analysis was conducted to see linear pattern (L) and quadratic pattern (Q) among the treatments. To calculate the optimal concentration of tannic acid in broilers, segmented linear regression was conducted, and *X0* (inflection point) with *P* values for slope 1 (lower than *X0*) and slope 2 (higher than *X0*) were presented.

4.3.4. Activities of jejunal brush border digestive enzymes and serum alkaline phosphatase

Activities of jejunal maltase, sucrase, APN, and IAP were not altered by supplemental TA in broilers (Figure 4.4.; $P > 0.1$). However, the activities of lipase showed a tendency to be reduced by TA at concentrations greater than 595.3 mg/kg ($P = 0.094$). Activities of alkaline phosphatase in the serum were not affected by supplemental TA (Figure 4.4.; $P > 0.1$).

4.3.5. Relative mRNA expression of genes related to nutrient transportation, gut barrier integrity, and inflammation in the jejunum

The relative mRNA expression of nutrient transporters, tight junction proteins, and cytokines in broilers fed different concentrations of TA is presented in Figure 4.5. No differences were observed in the mRNA expression of sodium-dependent neutral amino acid transporter 1 (B0AT1), excitatory amino acid transporter 3 (EAAT3), peptide transporter 1 (PepT1), junctional adhesion molecule 2 (JAM2), interleukins 1β (IL1β), and nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) ($P > 0.1$). Supplemental TA linearly ($P < 0.05$) and quadratically (tendency; $P = 0.088$) reduced mRNA expression of sodium glucose transporter 1 (SGLT1), and the TA1.5 group had significantly lower mRNA expression of SGLT1 compared to the TA0 group. The mRNA expression of mucin 2 (MUC2) was linearly decreased by TA at concentrations less than 784.9 mg/kg ($P < 0.05$). The mRNA expression of zonula occludens 2 (ZO2) was linearly and quadratically modulated ($P < 0.05$) and was linearly reduced by supplemental TA at concentrations less than 892.6 mg/kg ($P < 0.01$), and the TA1.5 group had significantly lower mRNA of ZO2 compared to the TA0 group. Supplemental TA linearly decreased mRNA expression of IL2 ($P < 0.05$) and IL6 (tendency; $P = 0.09$).

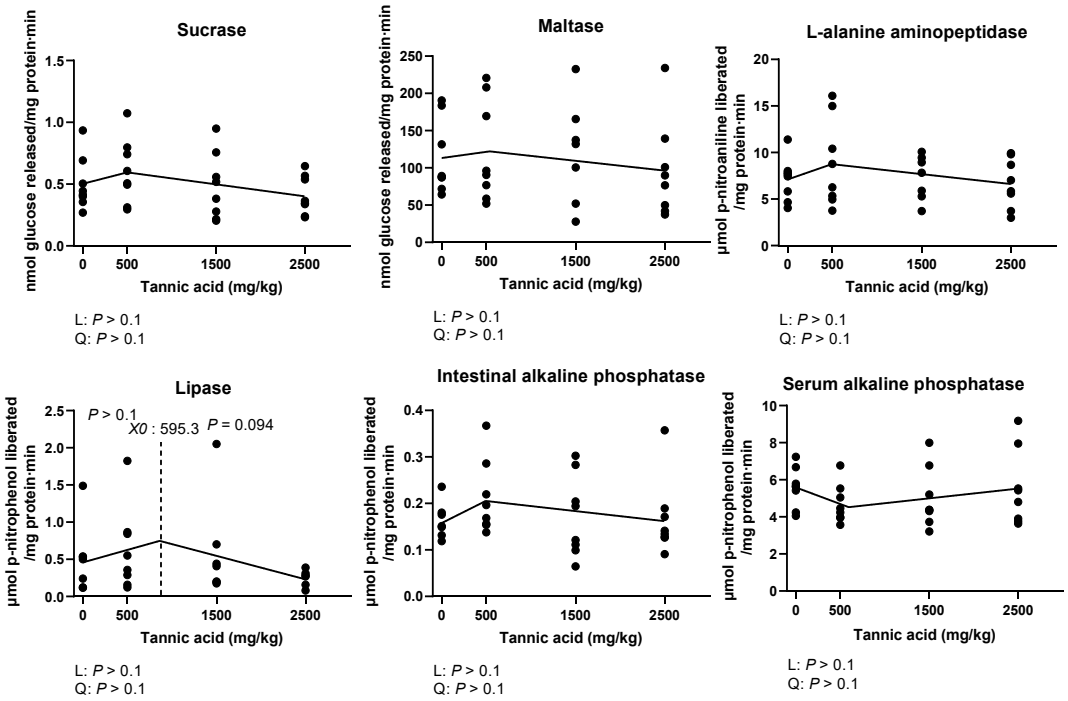


Figure 4.1. Activities of sucrase, maltase, L-alanine aminopeptidase, intestinal alkaline phosphatase, serum alkaline phosphatase lipase in the jejunum in broiler chickens fed different concentrations (0, 0.5, 1.5, and 2.5 g/kg) of tannic acid on D 21. The treatment groups were compared using one-way ANOVA followed by the Tukey's multiple comparison. Different superscripts differ at $P < 0.05$. Orthogonal polynomial contrasts analysis was conducted to see linear pattern (L) and quadratic pattern (Q) among the treatments. To calculate the optimal concentration of tannic acid in broilers, segmented linear regression was conducted, and *X0* (inflection point) with *P* values for slope 1 (lower than *X0*) and slope 2 (higher than *X0*) were presented.

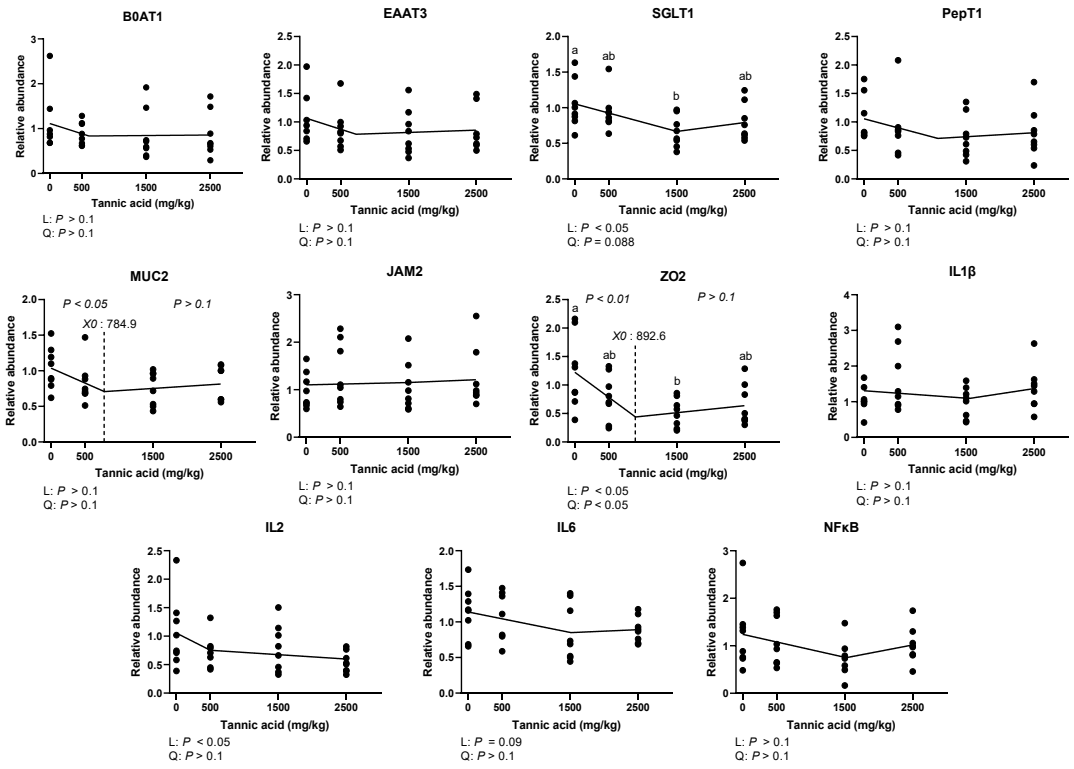


Figure 4.2. Relative mRNA expression of genes associated with nutrient transportation, gut barrier integrity, and inflammation in the jejunum in broiler chickens fed different concentrations (0, 0.5, 1.5, and 2.5 g/kg) of tannic acid on D 21. The treatment groups were compared using one-way ANOVA followed by the Tukey's multiple comparison. Different superscripts differ at $P < 0.05$. Orthogonal polynomial contrasts analysis was conducted to see linear pattern (L) and quadratic pattern (Q) among the treatments. To calculate the optimal concentration of tannic acid in broilers, segmented linear regression was conducted, and *X0* (inflection point) with *P* values for slope 1 (lower than *X0*) and slope 2 (higher than *X0*) were presented. B0AT1, sodium-dependent neutral amino acid transporter 1; EAAT3, excitatory amino acid transporter 3; SGLT1, sodium glucose transporter 1; PepT1, peptide transporter 1; MUC2, mucin 2; JAM2, Junctional adhesion molecule 2; Zonula occludens 2; zonula

occludens 2; IL, interleukin; NFκB, nuclear factor kappa-light-chain-enhancer of activated B cells.

4.3.6. Total antioxidant capacity, activities of superoxide dismutase, glutathione, and oxidized glutathione concentrations in the liver

Total antioxidant capacity was not modulated by supplemental TA (Figure 4.6.; $P > 0.1$). The TA0.5 group had the highest activities of SOD among the treatment groups ($P < 0.05$). Although there were no differences in total GSH, and reduced GSH, supplemental TA showed a tendency to linearly increase GSSG in broilers ($P = 0.069$) and linearly decreased reduced GSH:GSSG ($P < 0.05$).

4.3.7. Bone mineral density and body composition analyses

The bone mineral density and body composition indices in broilers fed different concentrations of TA are presented in Figure 4.7. Bone mineral density (tendency; $P = 0.05$) were linearly reduced as supplemental TA concentrations increased. Tissue weight was linearly reduced ($P < 0.05$) by supplemental TA, and TA2.5 had the lower tissue weight compared to the TA0 group ($P < 0.05$). Fat weight was linearly reduced ($P < 0.05$), and the TA2.5 group had lower fat weight compared to the TA0 group ($P < 0.05$). Body fat percentage and lean weight were significantly reduced by supplemental TA ($P < 0.05$). The lean to fat ratio was linearly increased as supplemental TA concentrations increased ($P < 0.05$).

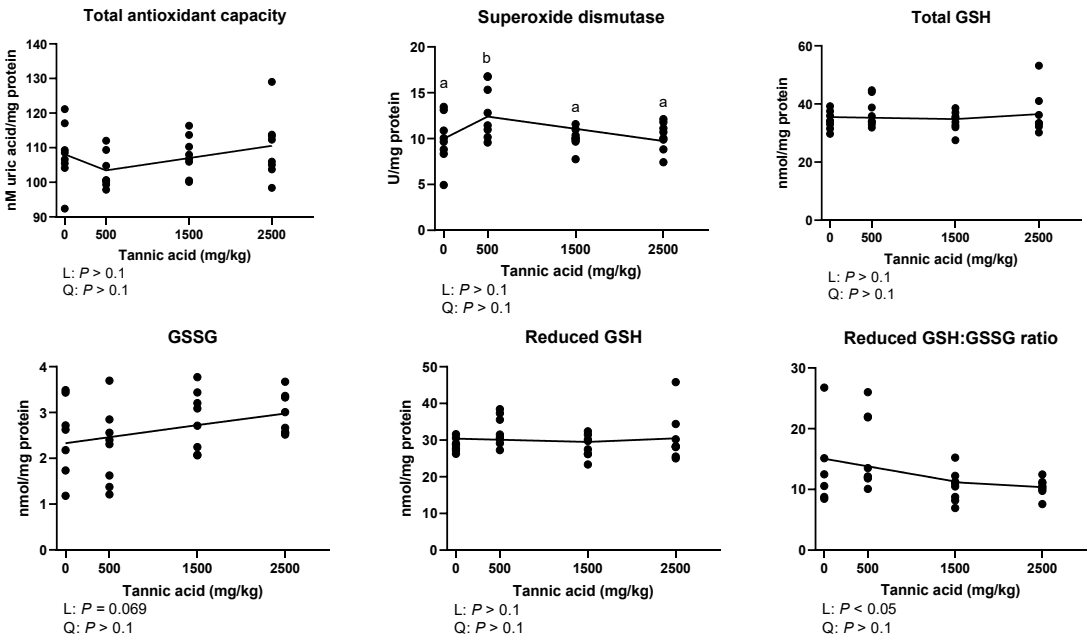


Figure 4.4. Total antioxidant capacity, glutathione and oxidized glutathione concentrations, and activities of superoxide dismutase in broiler chickens fed different concentrations (0, 0.5, 1.5, and 2.5 g/kg) of tannic acid on D 21. The treatment groups were compared using one-way ANOVA followed by the Tukey's multiple comparison. Different superscripts differ at $P < 0.05$. Orthogonal polynomial contrasts analysis was conducted to see linear pattern (L) and quadratic pattern (Q) among the treatments. To calculate the optimal concentration of tannic acid in broilers, segmented linear regression was conducted, and *X0* (inflection point) with *P* values for slope 1 (lower than *X0*) and slope 2 (higher than *X0*) were presented. Reduced GSH = Total GSH – 2 × GSSG.

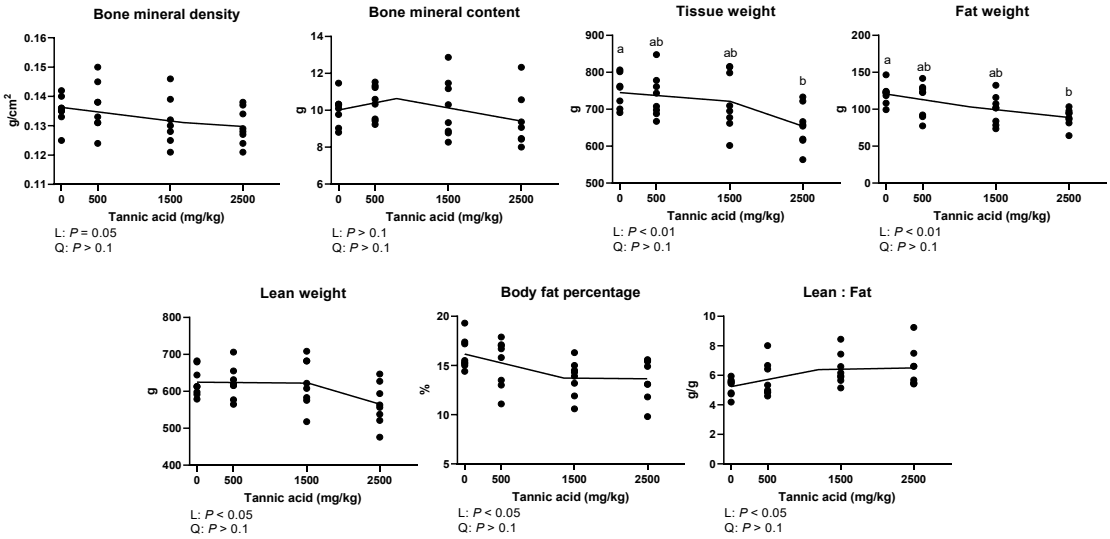


Figure 4.5. Bone mineral density (g/cm^2), bone mineral content (g), tissue weight (g), fat weight (g), lean weight (g), body fat percentage (%), and lean:fat (g/g) in broiler chickens fed different concentrations (0, 0.5, 1.5, and 2.5 g/kg) of tannic acid on D 21. The treatment groups were compared using one-way ANOVA followed by the Tukey's multiple comparison. Different superscripts differ at $P < 0.05$. Orthogonal polynomial contrasts analysis was conducted to see linear pattern (L) and quadratic pattern (Q) among the treatments. To calculate the optimal concentration of tannic acid in broilers, segmented linear regression was conducted, and *X0* (inflection point) with *P* values for slope 1 (lower than *X0*) and slope 2 (higher than *X0*) were presented.

4.3.8. Relative mRNA expression of genes related to fat metabolism in the liver

Supplemental TA did not modulate mRNA expression of genes related to fat metabolism in the liver of broilers (Figure 4.8.; $P > 0.1$)

4.3.9. Cecal volatile fatty acid composition

Modulated cecal VFA composition in broilers fed different concentrations of TA in Figure 4.9. The acetate concentration tended to be reduced at TA concentrations greater than 1,037 mg/kg were supplemented ($P = 0.072$). Supplemental TA linearly decreased the concentrations of butyrate and valerate ($P < 0.05$). Total VFA concentrations tended to be reduced by TA at concentrations greater than 850.9 mg/kg ($P = 0.073$).

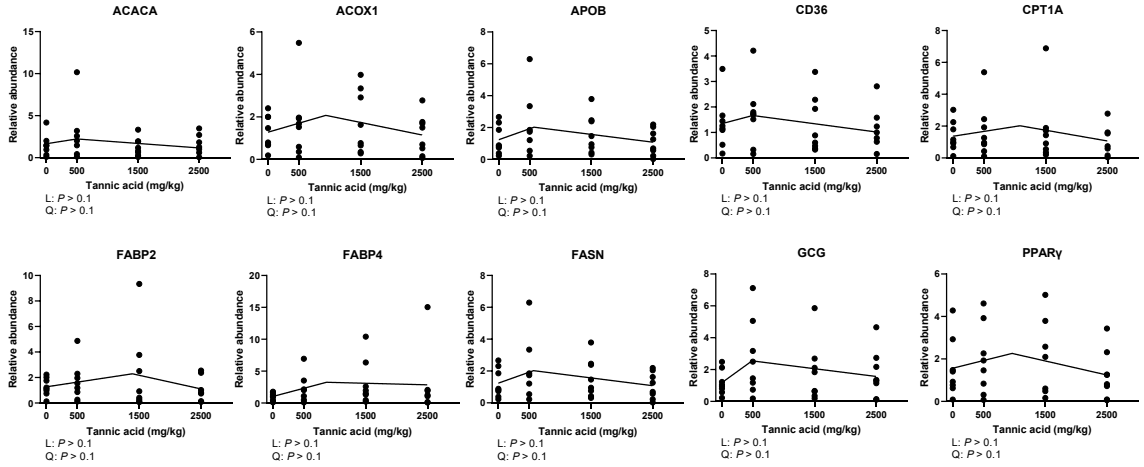


Figure 4.6. Relative mRNA expression of genes related to fat metabolism in the liver of broiler chickens fed different concentrations (0, 0.5, 1.5, and 2.5 g/kg) of tannic acid on D 21. The treatment groups were compared using one-way ANOVA followed by the Tukey's multiple comparison. Different superscripts differ at $P < 0.05$. Orthogonal polynomial contrasts analysis was conducted to see linear pattern (L) and quadratic pattern (Q) among the treatments. To calculate the optimal concentration of tannic acid in broilers, segmented linear regression was conducted, and *X0* (inflection point) with *P* values for slope 1 (lower than *X0*) and slope 2 (higher than *X0*) were presented. ACACA, acetyl-CoA carboxylase alpha; ACOX1, acyl-CoA oxidase 1; APOB, apolipoportine B; CD36, clusters of differentiation 36; CPT1A, carnitine palmitoyltransferase 1A; FABP, fatty acid binding protein; FASN, fatty acid synthase; GCG, glucacon; PPARγ, peroxisome proliferator-activated receptor gamma

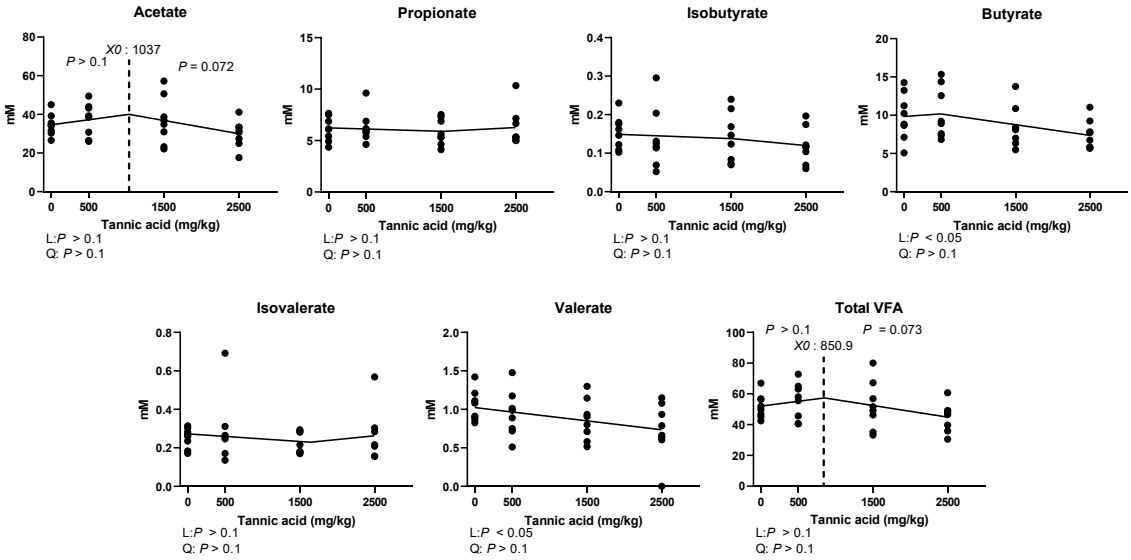


Figure 4.7. Cecal volatile fatty acid concentration (mM) in broiler chickens fed different concentrations (0, 0.5, 1.5, and 2.5 g/kg) of tannic acid on D 21. The treatment groups were compared using one-way ANOVA followed by the Tukey's multiple comparison. Different superscripts differ at $P < 0.05$. Orthogonal polynomial contrasts analysis was conducted to see linear pattern (L) and quadratic pattern (Q) among the treatments. To calculate the optimal concentration of tannic acid in broilers, segmented linear regression was conducted, and *X0* (inflection point) with *P* values for slope 1 (lower than *X0*) and slope 2 (higher than *X0*) were presented.

4.3.10. Alpha and beta diversity of the cecal microbial communities

The alpha diversity indices including pielou evenness, faith phylogenetic diversity, observed features, and shannon entropy are presented in Figure 4.10. Supplemental TA tended to linearly decrease pielou evenness (P = 0.1). Faith phylogenetic diversity tended to be reduced by TA at concentrations greater than 1,248 mg/kg (P = 0.07). However, there were no differences in observed features and shannon entropy.

Beta diversity indices including weighted and unweighted unifrac are presented in Figure 4.11. No statistical differences were observed in those indices. Visualized beta diversity indices such as weighted emperor, unweighted emperor, bray curtis, and jaccard did not show any obvious differences (Figure 4.12.).

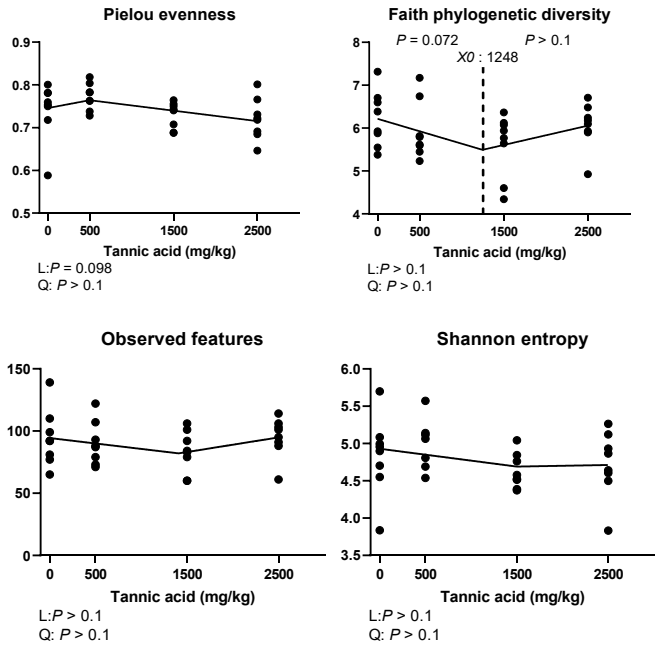


Figure 4.8. Alpha diversity indices of the cecal microbial communities in broiler chickens fed different concentrations (0, 0.5, 1.5, and 2.5 g/kg) of tannic acid on D 21. The treatment groups were compared using one-way ANOVA followed by the Tukey's multiple comparison. Different superscripts differ at $P < 0.05$. Orthogonal polynomial contrasts analysis was conducted to see linear pattern (L) and quadratic pattern (Q) among the treatments. To calculate the optimal concentration of tannic acid in broilers, segmented linear regression was conducted, and *X0* (inflection point) with *P* values for slope 1 (lower than *X0*) and slope 2 (higher than *X0*) were presented.

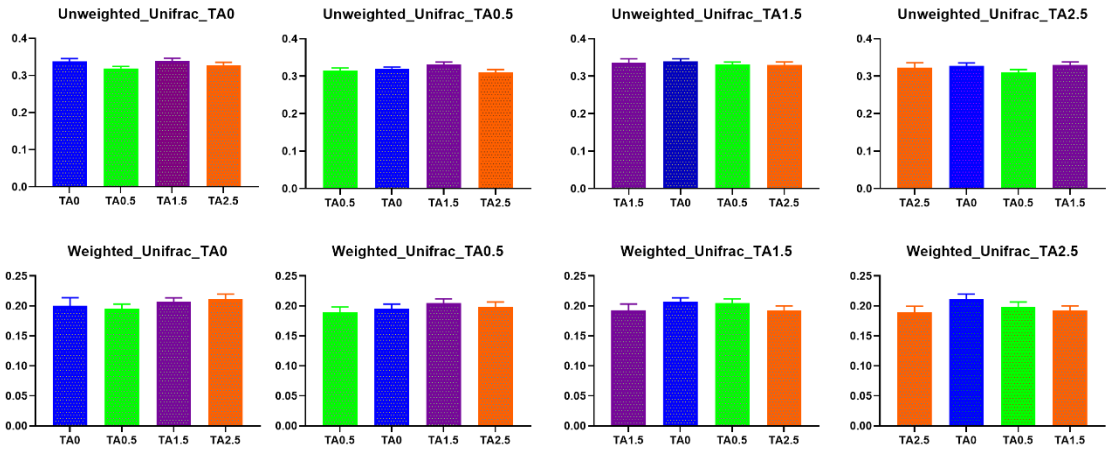


Figure 4.9. Beta diversity indices (weighted and unweighted unifrac) of the cecal microbial communities in the broilers of the tannic acid 0 (TA0): basal diet without tannic acid; 2) tannic acid 0.5 (TA0.5): basal diet with 0.5 g/kg tannic acid; 3) tannic acid 1.5 (TA1.5); and 4) tannic acid 2.5 (TA2.5) groups on D 21. The treatment groups were compared using one-way ANOVA followed by the Tukey's multiple comparison. Different superscripts differ at $P < 0.05$.

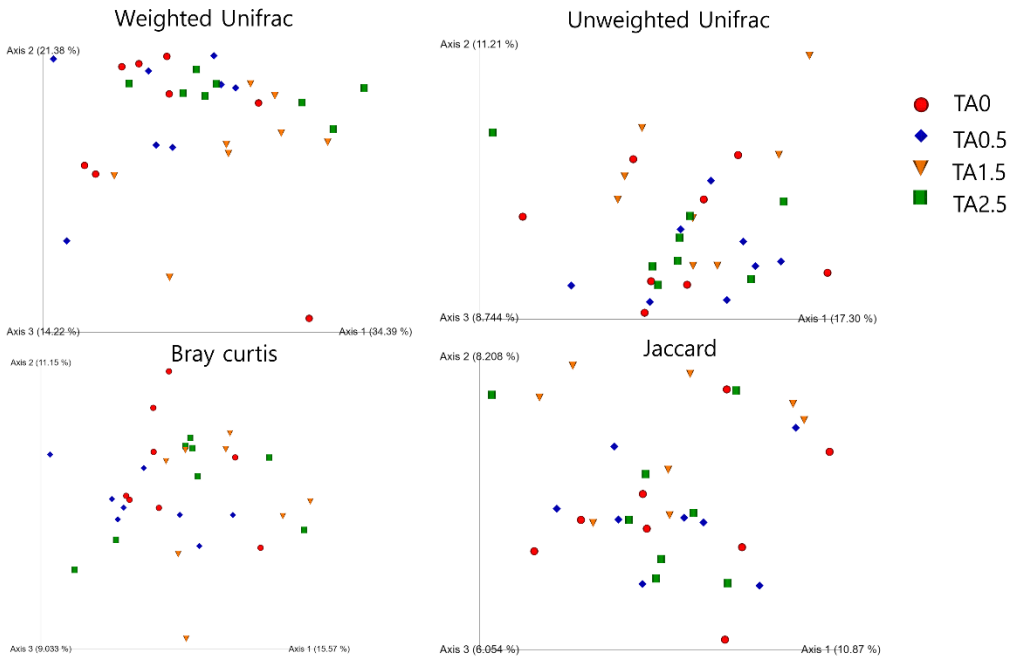
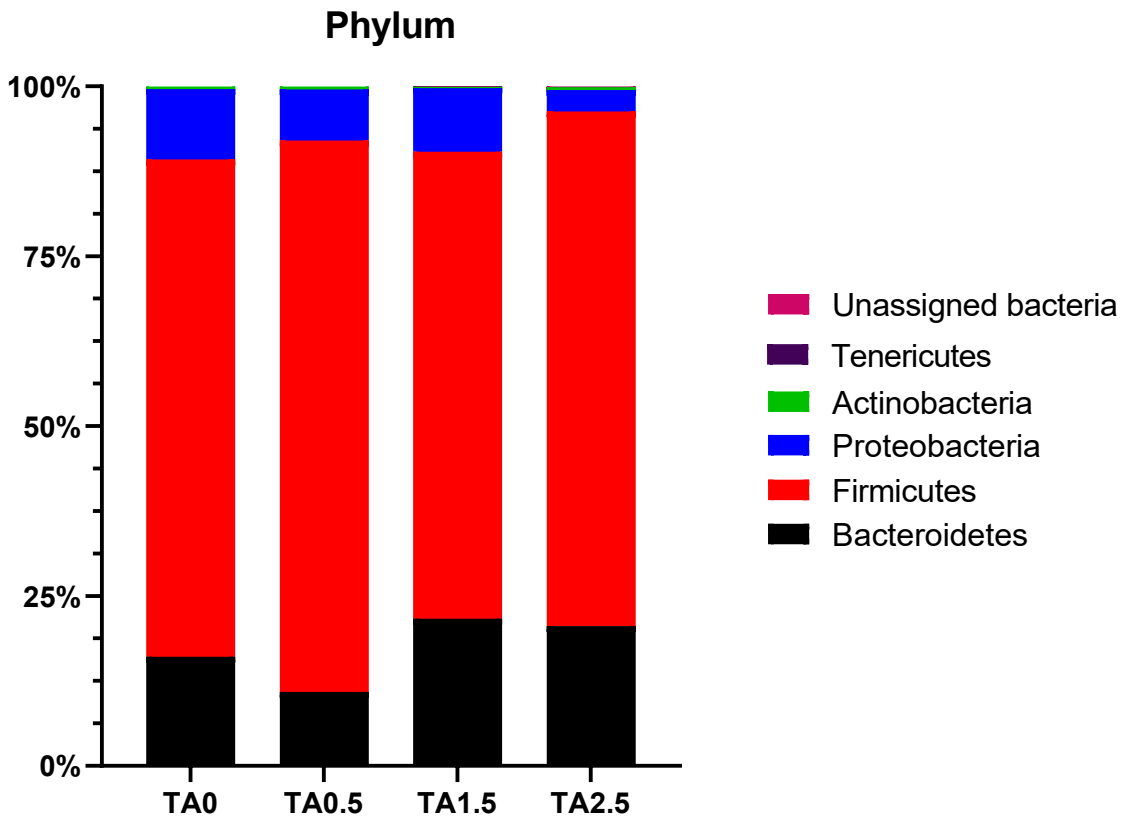
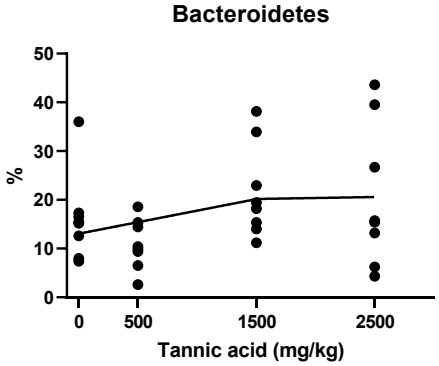


Figure 4.10. Visualization of beta diversity indices (weighted and unweighted unifrac, bray curtis, and jaccard) in in the broilers of the tannic acid 0 (TA0): basal diet without tannic acid; 2) tannic acid 0.5 (TA0.5): basal diet with 0.5 g/kg tannic acid; 3) tannic acid 1.5 (TA1.5); and 4) tannic acid 2.5 (TA2.5) groups on D 21.

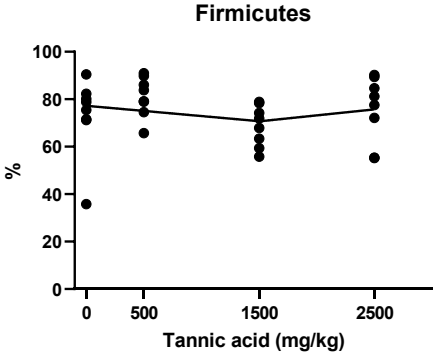
4.3.11. Phylum- and family-level composition of the cecal microbial communities

The phylum level composition of the cecal microbial communities is shown in Figure 4.13. The relative abundance of the phylum Bacteroidetes tended to be increased by supplemental TA ($P = 0.098$), whereas the relative abundance of the phylum Proteobacteria tended to be decreased by supplemental TA ($P = 0.097$). Family level composition of the cecal microbial communities is presented in Figure 4.14. The relative abundance of the family Rikenellaceae tended to be linearly reduced by TA at levels less than 500 mg/kg ($P = 0.05$) and tended to be linearly increased at levels greater than 500 mg/kg ($P = 0.1$). The relative abundance of unclassified Clostridiales was linearly decreased ($P < 0.05$) by supplemental TA. The relative abundance of the family Bacillaceae was quadratically modulated ($P < 0.05$) and linearly reduced at levels less than 1,045 mg/kg ($P < 0.05$) and tended to be linearly increased at levels greater than 1,045 mg/kg ($P = 0.06$). The heatmap visually presents differences at all levels in the composition of cecal microbial communities by supplemental TA (Figure 4.15.).

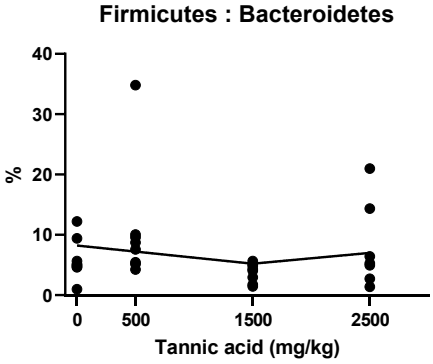




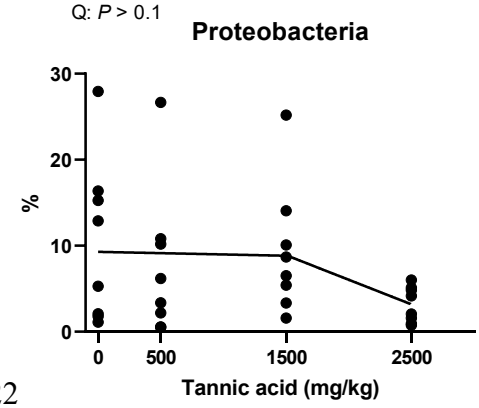
L: P = 0.098
Q: P > 0.1



L: P > 0.1
Q: P > 0.1



L: P > 0.1
Q: P > 0.1



L: P = 0.097
Q: P > 0.1

Figure 4.11. Phylum-level composition of the cecal microbial communities in broiler chickens fed different concentrations (0, 0.5, 1.5, and 2.5 g/kg) of tannic acid on D 21. Only phylum with significant differences and the relatively high relative abundance were presented separately with statistical analyses. The treatment groups were compared using one-way ANOVA followed by the Tukey's multiple comparison. Different superscripts differ at $P < 0.05$. Orthogonal polynomial contrasts analysis was conducted to see linear pattern (L) and quadratic pattern (Q) among the treatments. To calculate the optimal concentration of tannic acid in broilers, segmented linear regression was conducted, and *X0* (inflection point) with *P* values for slope 1 (lower than *X0*) and slope 2 (higher than *X0*) were presented.

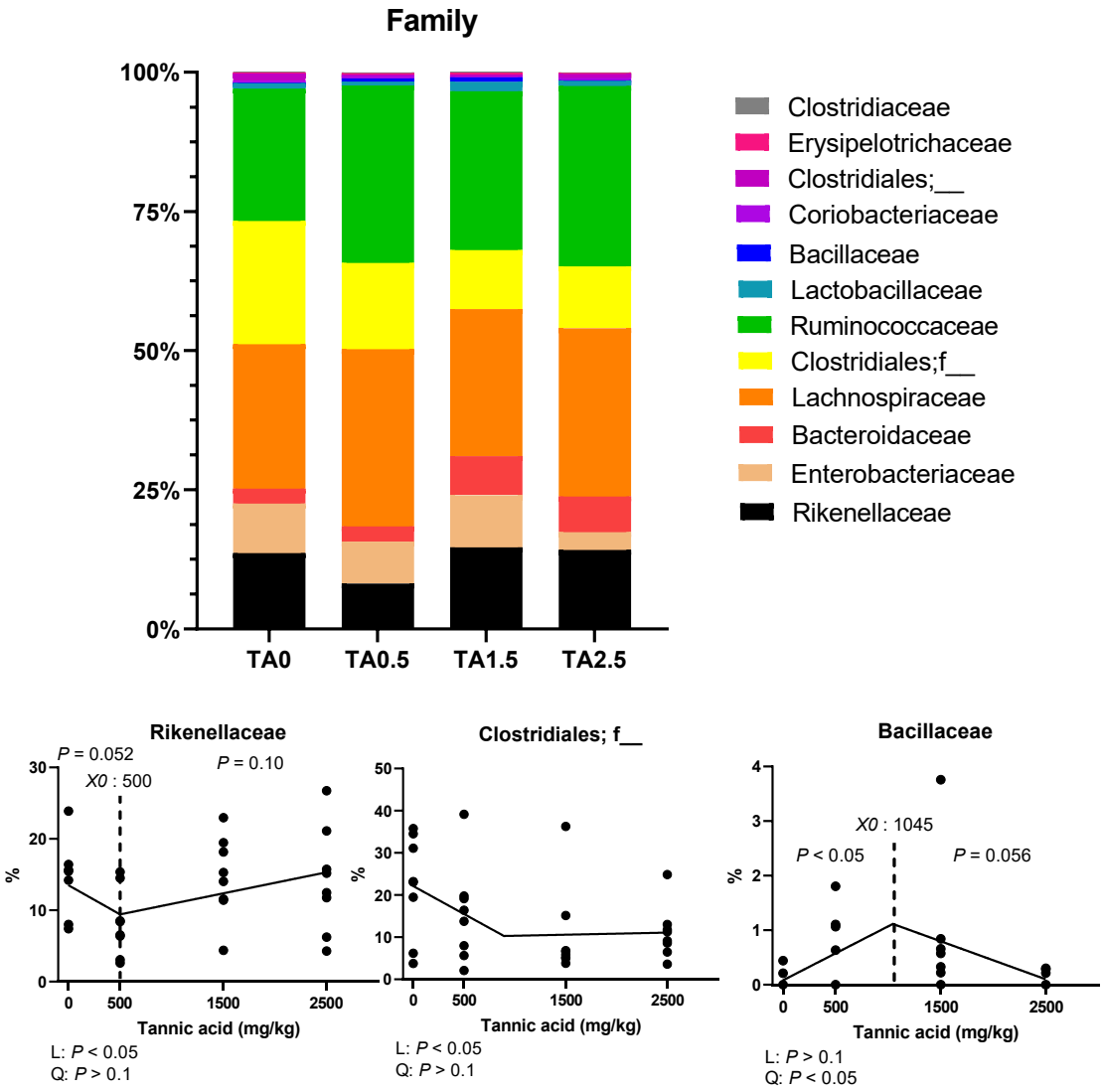


Figure 4.12. Phylum-level composition of the cecal microbial communities in broiler chickens fed different concentrations (0, 0.5, 1.5, and 2.5 g/kg) of tannic acid on D 21. Only phylum with significant differences were presented separately with statistical analyses. The treatment groups were compared using one-way ANOVA followed by the Tukey's multiple comparison. Different superscripts differ at $P < 0.05$. Orthogonal polynomial contrasts analysis was conducted to see linear pattern (L) and quadratic pattern (Q) among the treatments. To calculate

the optimal concentration of tannic acid in broilers, segmented linear regression was conducted, and *X0* (inflection point) with *P* values for slope 1 (lower than *X0*) and slope 2 (higher than *X0*) were presented.

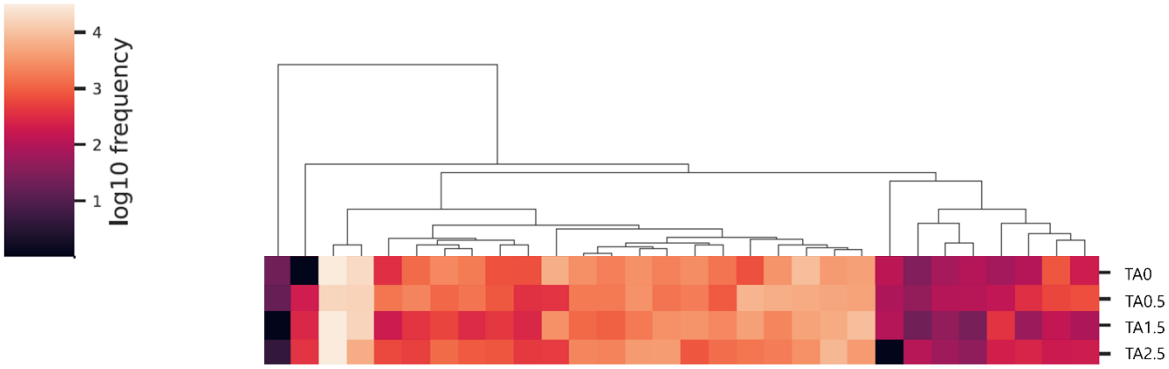


Figure 4.13. Heatmap of cecal microbiome in the broilers of the tannic acid 0 (TA0): basal diet without tannic acid; 2) tannic acid 0.5 (TA0.5): basal diet with 0.5 g/kg tannic acid; 3) tannic acid 1.5 (TA1.5): basal diet with 1.5 g/kg tannic acid; and 4) tannic acid 2.5 (TA2.5): basal diet with 2.5 g/kg tannic acid groups on D 21.

4.3.12. Correlation between the cecal microbial composition with volatile fatty acid production and parameters of growth performance, fat accumulation, bone health, fat metabolism mRNA expression, apparent ileal digestibility, immune system, gut barrier integrity, brush border digestive enzymes, nutrient transporters, antioxidant capacity, and volatile fatty acid production

The cecal microbial composition and VFA were positively or negatively correlated with diverse parameters of growth performance, fat accumulation, bone health, fat metabolism mRNA expression, AID, brush border digestive enzyme activities, nutrient transporters, immune system, gut barrier integrity, antioxidant capacity, and VFA production (Table 4.3.; $P < 0.05$). To be specific, the phylum Bacteroidetes was negatively correlated with body fat percentage ($P < 0.05$; $R^2 = -0.368$) and SOD activities ($P < 0.05$; $R^2 = -0.411$). The phylum Firmicutes were positively correlated with EAAT3 ($P < 0.05$; $R^2 = -0.358$), SGLT1 ($P < 0.05$; $R^2 = -0.429$), and JAM2 ($P < 0.05$; $R^2 = -0.36$). The order Clostridiales were positively correlated with PepT1 ($P < 0.05$; $R^2 = 0.366$), MUC2 ($P < 0.05$; $R^2 = 0.365$), and NFκB ($P < 0.05$; $R^2 = 0.365$). The family Bacillaceae was positively correlated with jejunal lipase activities ($P < 0.05$; $R^2 = 0.358$), cecal acetate production ($P < 0.05$; $R^2 = 0.504$), cecal VFA production ($P < 0.05$; $R^2 = 0.457$) but negatively correlated with AID of CP ($P < 0.05$; $R^2 = 0.358$). The cecal acetate concentration was positively correlated with jejunal sucrase activities ($P < 0.05$; $R^2 = 0.428$), jejunal APN activities ($P < 0.05$; $R^2 = 0.43$), jejunal IAP activities ($P < 0.05$; $R^2 = 0.406$) but negatively correlated with AID of DM ($P < 0.05$; $R^2 = -0.368$) and AID of OM ($P < 0.05$; $R^2 = -0.371$). Total VFA was positively correlated with jejunal sucrase activities ($P < 0.05$; $R^2 = 0.395$), jejunal APN activities ($P < 0.05$; $R^2 = 0.414$), jejunal IAP

activities ($P < 0.05$; $R^2 = 0.401$) but negatively correlated with liver TAC ($P < 0.05$; $R^2 = -0.364$), AID of DM ($P < 0.05$; $R^2 = -0.369$) and AID of OM ($P < 0.05$; $R^2 = -0.37$).

Table 4.3. Correlation between the cecal microbial composition with volatile fatty acids and growth performance (ADFI), fat accumulation (fat %), bone health (BMC and BMD), fat metabolism mRNA expression (ACOX1, APOB, CPT1A, FABP2, FABP4, FASN, and PPARγ), immune system (IL1β), gut barrier integrity (JAM2 and MUC2), brush border digestive enzymes (APN, Lipase, Sucrase, Maltase, and IAP), antioxidant capacity (TAC and SOD), apparent ileal digestibility (DM, OM, and CP), nutrient transporters (EAAT3, B0AT1, SGLT1, and PepT1), and volatile fatty acid production parameters (acetate, propionate, and isobutyrate) with significant differences ($P < 0.05$) in broilers fed different concentration of tannic acid on D 21.

	Items	*P* value	R^2	Items	*P* value	R^2	Items	*P* value	R^2	Items	*P* value	R^2
Bacteroidetes	Fat %	0.042	-0.368	CPT1A	0.023	0.401	SOD	0.022	-0.411			
Actinobacteria	FABP2	0.012	0.440									
Proteobacteria	Lipase	0.042	0.367									
Firmicutes	EAAT3	0.048	0.358	SGLT1	0.016	0.429	JAM2	0.047	0.360			
Actinobacteria	IL1β	0.019	0.420	Isobutyrate	0.046	-0.361						
Tenericutes	IL1β	0.037	0.377									
Enterobacteriaceae	Lipase	0.026	0.399									
Bacteroidaceae	ADFI	0.044	-0.364	ACOX1	0.007	0.470	CPT1A	0.003	0.507	PPARγ	0.003	0.506
	Propionate	0.017	0.426									

Lachnospiraceae	BMD	0.019	0.420	BMC	0.048	0.358	Valerate	0.013	-0.440			
Clostridiales;f__	PepT1	0.043	0.366	MUC2	0.044	0.365	NFκB	0.048	0.358			
Ruminococcaceae	IAP	0.014	0.439	NFκB	0.042	-0.368						
Lactobacillaceae	ADFI	0.003	0.513	Lipase	0.016	0.430	NFκB	0.028	-0.394	SOD	0.006	-0.513
	Ash	0.006	0.481									
Bacillaceae	PPARγ	0.020	0.409	Lipase	0.048	0.358	CP	0.049	-0.357	Acetate	0.004	0.504
	Total VFA	0.010	0.457									
Coriobacteriaceae	FABP2	0.009	0.456	FABP4	0.047	0.354	IL1β	0.024	0.405	Isobutyrate	0.041	-0.369
Clostridiales;__	B0AT1	0.001	0.549	EAAT3	0.016	0.428	SGLT1	0.017	0.425	NFκB	0.011	0.449
Clostridiaceae	PepT1	0.012	0.446	MUC2	0.032	0.386	IL1β	0.005	0.490	DM	0.002	-0.541
	OM	0.002	-0.535	Ash	0.010	-0.457	CP	0.006	-0.484			
Erysipelotrichaceae	ACOX1	0.001	0.542	APOB	0.003	0.507	FABP4	0.004	0.491	FASN	0.003	0.51
Acetate	Sucrase	0.016	0.428	APN	0.016	0.430	IAP	0.024	0.406	DM	0.042	-0.368
	OM	0.040	-0.371									
Propionate	CPT1A	0.002	0.531	PPARγ	0.024	0.398	GSSG	0.026	0.400	Ash	0.010	-0.454
Isobutyrate	Maltase	0.032	0.385									
Butyrate	TAC	0.018	-0.422									
Isovalerate	Fat %	0.003	-0.517	Fat weight	0.021	-0.413	DM	0.014	-0.438	OM	0.017	-0.424
	Ash	0.005	-0.495	CP	0.023	-0.406						

Valerate	PPARγ	0.046	0.355	Ash	0.041	-0.370						
Total VFA	Sucrase	0.028	0.395	APN	0.021	0.414	IAP	0.025	0.401	TAC	0.044	-0.364
	DM	0.041	-0.369	OM	0.041	-0.370						

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4.4. DISCUSSION

The purposes of the study were to investigate the effects of supplemental TA on growth performance, gut health, antioxidant status, bone mineral density, body composition, and cecal VFA concentrations and microbiome in broilers and to calculate the optimal concentration of supplemental TA in broilers based on the parameters. In the current study, BW were linearly reduced as supplemental TA concentrations increased in the three phases (D 7, 14, and 21). Reduced BW in broiler fed supplemental TA on D 21 would be primarily due to reduced feed intake on D 7 to 14 because no statistical differences were observed in ADG, ADFI and FCR on D 21 in the current study. High concentrations of TA can reduce feed intake in broilers by causing irritation in esophagus and necrosis in crop, gizzard, and duodenum (Suleyman, 2017). The BW of broilers on D 21 started to be decreased when greater than 972 mg/kg of supplemental TA was offered to the birds, which implies that TA at concentrations less than 972 mg/kg can be used in broilers without compromising growth rate in broilers.

In the current study, AID of CP and EE were reduced by supplemental TA. Tannins are considered as anti-nutritional factors mainly because TA interacts with dietary and endogenous proteins (e.g., digestive enzymes), which impairs nutrient digestion and absorption in broilers (Ortiz et al., 1993). Because there were no statistical differences in the jejunal morphology, and APN activities and amino acid and peptide transporters (e.g., B0AT1, EAAT3, and PepT1) in the present study, interaction of TA with dietary proteins might have been the main factor that reduced AIP of CP in broilers. In the current study, AID of EE was decreased by supplemental TA in broilers along with reduced AID of CP. Reduced AID of EE might be closely associated with reduced lipase activities in the current study. The TA

potentially impaired activities of lipase by forming a complex with lipase (Moreno-Córdova et al., 2020). Moreover, TA may have interacted with dietary lipid sources and bile salts. Fan et al. (2013) showed that TA inhibited the production of lipid droplets, which can be an obstacle to be digested by the host. A previous study by Li et al. (2019c) reported that condensed tannins can precipitate bile salts that play an important role in facilitating lipid digestions. Along with decreased AID of CP and EE and mRNA expression of the glucose transporter (SGLT1), supplemental TA possibly reduced the AID of gross energy in the current study. Reduce AID of nutrients may have negatively affected growth performance in broilers in the current study.

Intestinal mucus, which is produced by goblet cells, play important roles in facilitating nutrient digestion and absorption, protecting the enterocytes, and preventing invasion of pathogens (Duangnumsawang et al., 2021). Goblet cells are derived from multipotent stem cells near the base of the crypts, and immature goblet cells starts to rapidly synthesize and secret mucus (Kim and Khan, 2013). In the current study, supplemental TA linearly reduced goblet cells in the crypts of the jejunum in broilers. This may have been closely associated with reduced AID of CP because certain amino acids (threonine, serine, and proline) are required to produce mucus efficiently (Koo et al., 2020). Deficiency of these amino acids may have disturbed goblet cell proliferation and mucus production. Consequently, reduced mucus production by supplemental TA potentially impaired nutrient digestion and absorption. Sicard et al. (2017) reported that pathogenic bacteria need intestinal mucus for their colonization. However, it is unclear whether reduced mucus production by supplemental TA at concentrations greater than 784.9 mg/kg can be beneficial for broilers infected with pathogens (e.g., *Clostridium perfringens*) because some pathogens can use mucus for colonization. Thus,

further studies are necessary to understand mucus production and pathogen colonization in broilers under bacterial infection conditions.

The tight junction proteins, such as JAM2 and ZO2, play an important role in maintaining gut barrier integrity (Suzuki, 2020). In the current study, the relative mRNA expression of ZO2 was reduced by supplemental TA, which suggests that supplemental TA can impair gut barrier integrity in broilers. Moreover, relative mRNA expression of MUC2, which is associated with gut barrier integrity, was reduced in this study. In contrast, many studies showed that supplemental TA improved gut barrier integrity in pigs (Song et al., 2021; Yu et al., 2020) and mice (Scaldaferri et al., 2014) in challenging conditions (e.g., weaning and intestinal bowel diseases) potentially via showing antimicrobial, anti-inflammatory and antioxidative effects. Although the current study showed that supplemental TA impaired gut barrier integrity in broilers without any challenging conditions, it is unknown how TA supplementation would affect gut barrier integrity in broilers under challenge conditions. Therefore, more future studies are required to investigate the beneficial effects of supplemental TA on gut barrier integrity in broilers in challenge conditions including pathogenic infection, heat stress, etc.

A previous study by Marzo et al. (2002) reported that high concentrations of dietary TA supplementation (25 g/kg) impaired liver functions in broilers by showing proteolytic activities. Our current study showed that TA supplementation (0 to 2.5 g/kg) linearly increased GSSG and decreased the ratio of reduced GSH and GSSG, which implies that supplemental TA may induced oxidative stress in the liver. Although TA is known to have strong antioxidative capacity in *in vitro* conditions (Kim et al., 2010), TA can cause oxidative stress

in the liver by exhibiting proteolytic activities. However, the TA0.5 group had the highest activities of SOD, an endogenous enzymatic antioxidant (Albarran et al., 2001), among the treatments in the present study. Therefore, TA supplementation can modulate endogenous antioxidant capacity in broilers.

In the current study, the modulation of microbiota and their activities by supplemental TA reduced concentrations of acetate, butyrate, valerate, and total VFA in the cecal contents of broilers. The VFA are important microbial metabolites and energy sources for gut health and growth in broilers (Choi et al., 2021); thus poor production of VFA can result in a reduction in growth performance and fat accumulation (Fluitman et al., 2017). The present study showed that concentrations of propionate and valerate were positively correlated to CPT1A ($P < 0.05$; $R^2 = 0.531$) and PPARγ ($P < 0.05$; $R^2 = 0.355$), respectively. This suggests that VFA production can potentially affect fat metabolism in chickens fed TA. In addition, VFA are also important energy sources for the development of enterocytes in the intestine of broilers (Bergman, 1990). In the current study, total VFA, acetate, and isobutyrate were positively correlated with brush border digestive enzymes (sucrase, APN, and IAP) which implies that VFA are important to induce gut development in chickens fed TA.

In the present study, supplemental TA tended to linearly reduce alpha diversity indices (diversity within a sample) including species equitability, expressed as pielou evenness, and faith phylogenetic diversity index in the cecal microbial communities of broilers. As the name refers, the pielou evenness was for measuring the evenness of microbial communities, and faith phylogenetic diversity was for measuring biodiversity based on phylogeny (González-Mercado et al., 2020). While it is still controversial, lower alpha diversity in cecal microbial

communities may indicate less stable and undeveloped microbial community compared to the higher alpha diversity (Choi et al., 2015). However, a previous study by Diaz Carrasco et al. (2018) reported that 1 g/kg supplemental TA increased shannon's diversity in broilers on D 30. The difference would be originated from different tannins sources [blend of tannins derived from chestnut (*Castanea sativa*) and quebracho (*Schinopsis lorentzii*; condensed tannins)] and different age of the broilers. No differences were observed in the beta diversity (diversity among treatments) in the current study.

In the present study, supplemental TA increased the relative abundance of the phylum Bacteroidetes in broilers. The phylum Firmicutes and the phylum Bacteroidetes are the main bacterial groups in the ceca of broilers (Wang et al., 2021a), and their relative abundance and ratio are closely associated with fat accumulation and nutrient absorption (Singh et al., 2013). Our current study showed that the relative abundance of the phylum Bacteroidetes was negatively correlated with body fat percentage ($P < 0.05$, $R^2 = - 0.368$), and the relative abundance of the phylum Firmicutes were positively correlated with EAAT3 ($P < 0.05$, $R^2 = 0.538$) and SGLT1 ($P < 0.05$, $R^2 = 0.429$) in broilers fed TA. Moreover, the phylum Bacteroidetes were negatively correlated with the activities of SOD ($P < 0.05$, $R^2 = - 0.411$), and the phylum Firmicutes were positively correlated with mRNA expression of JAM2 ($P < 0.05$, $R^2 = 0.360$) in broilers fed TA. This implies that supplemental TA negatively affected microbiota of broilers for better growth performance and fat accumulation. However, the relative abundance of the phylum Proteobacteria, which includes many pathogenic bacteria (*Escherichia coli*, *Salmonella* spp., *Vibrio*, etc) (Ma et al., 2017), were reduced by supplemental TA. Reduced relative abundance of the phylum Proteobacteria may indicate

better intestinal health and less pathogenic bacteria in broilers (Prasai et al., 2016). This would be due to strong antimicrobial effects of TA against certain pathogens such as *Salmonella* spp., *Escherichia coli* (Kim et al., 2010). Reduced the relative abundance of the phylum Proteobacteria may be closely associated with decreased relative mRNA expression of IL2 and IL6, proinflammatory cytokines, which indicates that TA supplementation potentially reduces inflammation by exhibiting antimicrobial effects in broilers. Based on antimicrobial and anti-inflammatory effects of TA, TA supplementation can be beneficial in broilers infected with pathogens in antibiotic-free conditions.

Pham et al. (2020) reported that the relative abundance of the family Rikenellaceae has been positively correlated with gastrointestinal tract dysfunctions. In the current study, the relative abundance of the family Rikenellaceae was decreased by dietary TA at concentrations less than 500 mg/kg and increased at concentrations greater than 500 mg/kg, which indicates that 500 mg/kg supplemental TA can be beneficial for gut health, whereas higher than 500 mg/kg supplemental TA can be detrimental for gut health of broilers. Supplemental TA reduced relative abundance of the unclassified Clostridiales in the current study, and its relative abundance was positively correlated with PepT1 ($P < 0.05$; $R^2 = 0.546$), MUC2 ($P < 0.05$; $R^2 = 0.365$), and NFκB ($P < 0.05$; $R^2 = 0.358$). Torok et al. (2011) also showed that the order Clostridiales are known to be positively correlated with growth performance. Our current study showed that the relative abundance of the family Bacillaceae peaked at 1,045 mg/kg supplemental TA. The relative abundance of the family Bacillaceae were positively correlated with acetate (main VFA) ($P < 0.05$; $R^2 = 0.504$), and total VFA ($P < 0.05$; $R^2 = 0.457$). In agreement, Wasti et al. (2021) reported that the relative abundance of the family Bacillaceae

was positively correlated with growth performance and VFA production. Based on the family concentration composition of cecal microbial communities, approximately 500 to 1,000 mg/kg of TA may promote a better gut microbial profile and VFA production.

In this study, bone mineral density and bone mineral content were reduced by supplemental TA, which indicates that supplemental TA reduced bone health in broilers. This result is in agreement with previous studies which reported that tannins can negatively affect bone health of broilers (Abbel-Monein, 2013; Tomaszewska et al., 2018). Tannins are known to inhibit the utilization of calcium, phosphorus, and iron by precipitation in the gastrointestinal tract, which can negatively affect bone development in animals (Hassan et al., 2003). However, there were no differences in AID of ash in the present study. More studies are required to investigate the direct effects of supplemental TA on bone development and maintenance in broilers.

In the current study, the fat accumulation and body fat percentage were reduced, and lean to fat ratio was increased by supplemental TA in broilers. These results indicate that the decrease in the fat accumulation was more pronounced compared to the reduction level in lean accumulation by supplemental TA in broilers. Reduced fat accumulation can be closely associated with reduced AID of EE, decreased activities of lipase, and modulated microbiota with VFA production in the current study. Liver was selected for fat metabolism analyses because liver is the main area for fat synthesis and metabolism in chickens (Zaefarian et al., 2019). However, mRNA expression of genes related to fat metabolism in the liver was not modulated by supplemental TA in the current study. Many studies have reported that TA modulated fat metabolism in the liver of the animals (Huang et al., 2019; Huang et al., 2013;

Park et al., 2002). Potentially, different alteration of lipid digestibility, VFA production, and microbiome by supplemental TA may have different impacts on the mRNA expression of genes associated with fat metabolism when the current study was compared to other studies. Many previous studies showed that dietary TA exhibit antinutritional effects mainly by interacting with dietary and endogenous proteins in broilers (Redondo et al., 2014a). In contrast, our current study also suggests that dietary TA had more severe effects on the fat accumulation rather than protein accumulation in broilers.

4.5. CONCLUSION

In summary, TA supplementation (0 to 2.5 g/kg) reduced growth performance, decreased nutrient utilization, increased lean to fat ratio, and negatively modulated microbiota in broilers. However, supplemental TA up to certain concentrations did not negatively influence growth performance at concentrations less than 972 mg/kg, lipase activities at concentrations less than 593.5 mg/kg, total VFA production at concentrations less than 850.9 mg/kg, and cecal microbiota at concentrations less than 1,045 mg/kg in broilers (Figure 4.16.). Furthermore, 500 mg/kg of supplemental TA increased activities of SOD in the liver and decreased the relative abundance of the family Rikenellaceae. Therefore, approximately supplementation of 500 to 1,000 mg/kg TA have potentials to be used as an AGP alternative in broilers under antibiotic-free conditions.

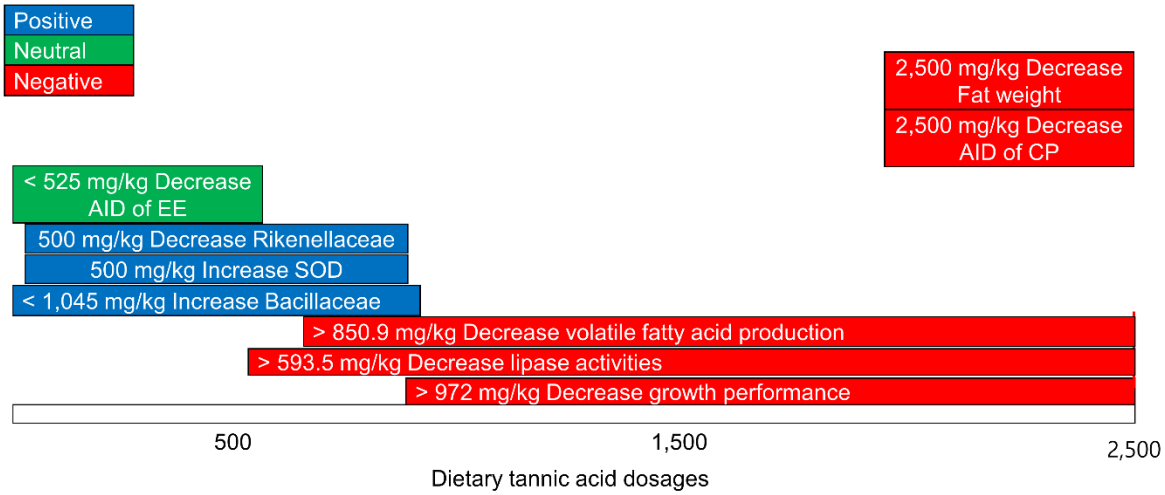

Figure 4.14. Summary of different effects of diverse doses of dietary tannic acid in broilers. SOD: superoxide dismutase; AID: apparent ileal digestibility, EE: ether extract; CP: crude protein

5.0. CHAPTER 5 MANUSCRIPT III

ANTIMICROBIAL AND IMMUNOMODULATORY EFFECTS OF TANNIC ACID SUPPLEMENTATION IN BROILERS INFECTED WITH SALMONELLA TYPHIMURIUM[1]

[1]**Choi, J.**, B. Marshall, H. Ko, H. Shi, A. K. Singh, H. Thippareddi, S. Holladay, R. M. Gogal Jr, and W. K. Kim. 2022. Antimicrobial and immunomodulatory effects of tannic acid supplementation in broilers infected with Salmonella Typhimurium. Poult. Sci.:102111. Reprinted here with permission of the publisher.

5.1. ABSTRACT

Infection by *Salmonella* Typhimurium, a food-borne pathogen, can reduce the poultry production efficiency. The objective of this study was to investigate the effects of tannic acid (TA) supplementation on growth performance, *Salmonella* colonization, gut barrier integrity, serum endotoxin concentrations, antioxidant capacity, gut health, and immune function in broilers infected with the *Salmonella enterica* serovar Typhimurium nalidixic acid resistant strain (ST^{NR}). A total of 546 one-day-old broilers were arbitrarily allocated into 6 treatments including 1) Sham-challenged control (SCC; birds fed a basal diet and administrated peptone water); 2) Challenged control (CC; birds fed a basal diet and inoculated with 10^8 ST^{NR}); 3) Tannic acid 0.25 (TA0.25; CC + 0.25 g/kg TA); 4) TA0.5 (CC + 0.5 g/kg TA); 5) TA1 (CC + 1 g/kg TA); and 6) TA2 (CC + 2 g/kg TA). On D 7, supplemental TA linearly reduced ST^{NR} colonization in the ceca ($P < 0.01$), and TA1 and TA2 group had significantly lower reduced ST^{NR} colonization in the ceca ($P < 0.01$). On D 7 to 21, average daily gain tended to be linearly increased by supplemental TA ($P = 0.097$). The serum endotoxin concentrations were quadratically decreased by supplemental TA on D 21 ($P < 0.05$). Supplemental TA quadratically increased ileal VH ($P < 0.05$), and the TA0.25 group had higher ileal VH compared to the CC group ($P < 0.05$). Supplemental TA linearly increased percentage of peripheral blood $CD8^+$ T cells on D 18 ($P < 0.01$). The TA0.5 group had significantly lower lymphocyte numbers compared to the CC groups ($P < 0.05$). The abundance of monocytes linearly increased with TA supplementation ($P < 0.01$). Therefore, broilers fed TA had reduced ST^{NR} colonization, increased growth performance, decreased serum endotoxin concentrations, enhanced gut health in the broilers, and stimulated the immune system in broilers infected with

ST^{NR}. TA supplementation (1 to 2 g/kg) enhanced growth performance and gut health via antimicrobial and immunostimulatory effects in broilers infected with ST^{NR}.

Keywords: Tannic acid, *Salmonella* Typhimurium, Antimicrobials, Immunity, Feed additive

5.2. INTRODUCTION

Salmonella enterica serovar Typhimurium is one of the most costly pathogens to poultry production because it threatens public health as a food-borne pathogen and reduces poultry production efficiency (Mughini-Gras et al., 2014). Because young broilers have an underdeveloped immune system, *S.* Typhimurium infection primarily impacts this age group, compromising their growth performance, gut health, and immunity (Alkie et al., 2019; Ibrahim et al., 2021). Impaired growth and gut health in the early broiler stage due to *S.* Typhimurium infection can negatively affect flock performance (Martínez et al., 2021). While *S.* Typhimurium infection in the older broilers can remain asymptomatic, *S.* Typhimurium contaminated poultry products can infect humans. This can induce severe gastrointestinal and systemic symptoms and even mortality in select susceptible human cohorts (Wilson et al., 2016). *S.* Typhimurium infection has been reported to cause 500,000 to 600,000 deaths in humans worldwide annually (Bula-Rudas et al., 2015).

To decrease the occurrence of *Salmonella* infection and to ameliorate the negative effects of *Salmonella* infection in broilers, antibiotic growth promotors (AGP) have been traditionally used in poultry production (Gut et al., 2018). However, due to the concern of spreading resistant bacteria and their resistant genes, some countries or institutions have restricted or strictly banned (EU) the use of AGP in poultry production (Abudabos et al., 2019). Therefore, cost-effective alternatives for AGP are required to control *Salmonella* infection and to improve growth performance in broiler production (Yang et al., 2015a).

Tannins, polyphenol compounds that can precipitate proteins, were recently considered as potential alternatives for AGP mainly due to their strong antimicrobial effects,

although tannins can show anti-nutritional effects at high doses in broilers (Choi and Kim, 2020). Tannic acid (TA) is considered as the standard of hydrolysable tannins, and chestnut TA is a by-product of the wood industry (Mannelli et al., 2019). In many *in vitro* studies, TA was shown to have strong antimicrobial effects against *Salmonella* spp. (Graziani et al., 2006; Sivasankar et al., 2020; Van Parys et al., 2010). A previous study by Ramah et al. (2020) reported that appropriate concentrations of TA enhanced broiler immunity. Therefore, the hypothesis of the study was that supplemental TA may improve growth performance and gut health in broilers infected with *S.* Typhimurium via its antimicrobial and immunomodulatory effects. The purpose of the study was to investigate the effects of supplemental TA on growth performance, *Salmonella* colonization, gut barrier integrity, serum endotoxin concentrations, liver and intestinal antioxidant capacity, ileal morphology, ileal brush border digestive enzymes activities, serum alkaline phosphatase activities, and peripheral immune system in broilers infected *S.* Typhimurium.

5.3. MATERIALS AND METHODS

5.3.1. Preparation of *Salmonella* Typhimurium inoculum

The inoculum of *Salmonella enterica* serovar Typhimurium nalidixic acid resistant strain (ST^{NR}) was prepared according to Yadav et al. (2022a). Briefly, a single colony of *in vitro* passaged ST^{NR} was cultured on the brilliant green sulfa (BGS; Difco, Detroit, MI) agar containing 200 μg/mL nalidixic acid (NA; Sigma-Aldrich Co., St Louis, MO). It was then streaked in tryptic soy broth (TSB; Sigma-Aldrich Co. St Louis, MO) containing 200 μg/mL of nalidixic acid (NA) and grown aerobically at 35 °C. After 24 h incubation, the culture was washed by centrifuging at 7,000 × *g* for 10 min and adding phosphate buffered saline (PBS)

twice. The bacterial cell optical density (OD) was measured at 600 nm (OD600) using a UV-Vis spectrometer (Genesys™ 10S UV-Vis, Thermo Fisher Scientific, Waltham, MA) to make 10^{10} per mL by adding peptone water (0.1%; Fisher Scientific, Fair Lawn, NJ) according to the standard curve generated previously. Afterwards, the 10^{10} CFU/mL bacterial solution was diluted to 10^{8} CFU/mL by adding peptone water.

5.3.2. Experimental design and growth performance

The study was reviewed and approved by the Institutional Animal Care and Use Committee at the University of Georgia, Athens, GA. A total of 546 one-day-old Cobb 500 broilers was randomly assigned to 6 treatment groups with 7 replicates of 13 birds. The 6 treatments included 1) Sham-challenged control (SCC; birds fed a basal diet and administrated peptone water); 2) Challenged control (CC; birds fed a basal diet and inoculated with 10^{8} ST^{NR}); 3) Tannic acid 0.25 (TA0.25; CC + 0.25 g/kg TA); 4) Tannic acid 0.5 (TA0.5; CC + 0.5 g/kg TA); 5) Tannic acid 1 (TA1; CC + 1 g/kg TA); and 6) Tannic acid 2 (TA2; CC + 2.0 g/kg TA). The basal diet was formulated to meet or exceed energy and nutrient requirements according to Cobb Broiler Management Guide (Table 5.1.; Cobb 2018). The TA (Sigma-Aldrich Co. St Louis, MO) was added into the filler part with sand to acquire the designated concentrations for each dietary treatment. Broilers were fed ad-libitum during the whole experimental period (21 D), and temperature and light were maintained according to Cobb Broiler Management Guide (Cobb 2018). One-day-old birds received either 0.5 mL peptone water for the SCC group or 0.5 mL of 10^{8} ST^{NR} in peptone water for the infected group. On D 7 and 21, body weight (BW) and feed disappearance were recorded to calculate average daily gain (ADG), average daily feed intake (ADFI), and feed conversion ratio (FCR).

Table 5.1. Ingredients and nutrient compositions of basal diets (As-fed basis)[1]

Items	D 0 to 7	D 7 to 21
Ingredients		
Corn	606.61	647.1
Soybean meal (480 g crude protein/kg)	330.76	291.18
Dicalcium phosphate	16.57	15.26
Filler[1]	10	10
Soybean oil	12.62	14.15
Limestone	11.7	11.11
DL-Methionine 99%	3.19	2.93
L-Lysine HCl 78%	2.51	2.5
Sodium chloride	3.5	3.51
L-threonine	1.2	0.77
Mineral Premix[3]	0.8	0.8
Vitamin Premix[2]	0.5	0.5
Choline Cl -70%	0.04	0.2
Total	1,000	1,000
Calculated energy and nutrient value, %		
Metabolizable energy, Mcal/kg	2,975	3,025
Crude protein	21.89	20.28
SID[4] Methionine	0.63	0.58
SID[4] Total sulfur amino acids	0.91	0.85
SID[4] Lysine	1.22	1.12
SID[4] Threonine	0.83	0.73
Total calcium	0.9	0.84
Available phosphate	0.45	0.42

[1] Sand and tannic acid were added to obtain desired tannic acid concentrations in the feed as follows: Sham-challenged control (SCC) and challenged control (CC): sand 10 g/kg + tannic acid 0 g/kg; Tannic acid 0.25 (TA0.25): sand 9.75 g/kg + tannic acid 0.25 g/kg; Tannic acid 0.5 (TA0.5): sand 9.5 g/kg + tannic acid 0.5 g/kg; Tannic acid 1 (TA1): sand 9 g/kg + tannic acid 1 g/kg; and Tannic acid 2 (TA2): sand 8 g/kg + tannic acid 2 g/kg. Tannic acid was purchased from Sigma–Aldrich (St Louis, MO).

[2] Vitamin mix provided the following in mg/100 g diet: thiamine-HCl, 1.5; riboflavin 1.5; nicotinic acid amide 15; folic acid 7.5; pyridoxine-HCl, 1.2; d-biotin 3; vitamin B-12 (source concentrations, 0.1%) 2; d-calcium pantothenate 4; menadione sodium bisulfite, 1.98; α-tocopherol acetate (source 500,000 IU/g), 22.8; cholecalciferol (source 5,000,000 IU/g) 0.09; retinyl palmitate (source 500,000 IU/g), 2.8; ethoxyquin, 13.34; I-inositol, 2.5; and dextrose, 762.2

[3] Mineral mix provided the following in g/100 g diet: $Ca(H_2PO_4)_2 \cdot H_2O$, 3.62; $CaCO_3$, 1.48; KH_2PO_4, 1.00; Na_2SeO_4, 0.0002; $MnSO_4 \cdot H_2O$, 0.035; $FeSO_4 \cdot 7H_2O$, 0.05; $MgSO_4 \cdot 7H_2O$, 0.62; KIO_3, 0.001; NaCl, 0.60; $CuSO_4 \cdot 5H_2O$, 0.008; $ZnCO_3$, 0.015; $CoCl_2 \cdot 6H_2O$, 0.00032; $NaMoO_4 \cdot 2H_2O$, 0.0011; KCl, 0.10; dextrose, 0.40.

[4] SID: standard ileal digestible

5.3.3. Colonization analysis of ceca, liver, and spleen and sample collection

On D 4, 7, 14, and 21, one bird per pen was euthanized via cervical dislocation, and the ceca, liver (without gall bladder), and spleen samples were collected using aseptic technique. Ceca samples were collected in sterile Whirl-Pak bags (Nasco, Fort Atkinson, WI), and liver and spleen samples were collected in the sterile sample bags (VWR International, Radnor, PA). The samples were stored in ice before being delivered to the lab. In the lab, bags containing the ceca were weighed and manually squeezed and homogenized using a paddle lab blender (Masticator Silver Panoramic, Neutec Group Inc, Farmingdale, NY) after adding 10 mL of buffered peptone water (BPW; HiMedia, Mumbai, India) containing 200 mg/kg NA. The cecal content homogenate was transferred to a 10 mL sterile glass tube and diluted to 10^{-6}. Next, 100 µL of diluted samples were plated on the prepared BGS agar containing 200 mg/kg NA followed by at 35 °C for 24 h. After the incubation, the number of ST^{NR} colonies was counted. To the sample bags containing liver and spleen samples, 10 mL and 5 mL of BPW containing 200 mg/kg NA were added, respectively and homogenized thoroughly by using hands and the paddle lab blender (Masticator Silver Panoramic, Neutec Group Inc). The homogenized liver and spleen samples were incubated at 35 °C for 24 h and plated on the BGS containing 200 mg/kg NA using sterile swabs and incubated at 35 °C for 24 h. Afterwards, the presence or absence of ST^{NR} was determined.

On D 7 and 21, mid-ileal tissue (half point of ileo-ceco-rectal junction and Meckel's diverticulum) samples were washed with PBS to remove digesta. For intestinal morphology analysis, ileal samples were immersed in 10% formalin for fixation. For analyses by real-time reverse transcription (RT)-PCR, intestinal antioxidant capacity, and ileal brush border

digestive enzyme activities, ileal tissue samples were snap-frozen in the liquid nitrogen. On D 21, liver samples were collected and snap-frozen in liquid nitrogen for liver antioxidant capacity analyses. Blood samples were collected on D 7 and 21 by heart puncture after euthanizing, and serum was recovered from clotted blood by centrifugation at 1,500 × *g* for 12 min. Serum samples and snap-frozen samples were stored at – 80 °C for further analyses.

5.3.4. Serum endotoxin concentrations and gut permeability analyses

Serum endotoxin concentrations were quantified using a Pierce™ LAL Chromogenic Endotoxin Quantitation Kit (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's protocol using a 1:9 dilution in endotoxin free water. On D 14 and 21, gut permeability was measured according to Choi et al. (2021). Briefly, 2.2 mg/mL fluorescein isothiocyanatedextran 4 kDa (FITC-D4; Sigma-Aldrich Co. St Louis, MO) were dissolved in PBS. One milliliter of the FITC solution was administrated to one bird per pen via oral gavage. After 2 h, birds were euthanized via cervical dislocation, and blood was collected from heart. The samples were stored in a dark container at room temperature to allow for clotting. Serum was recovered by centrifugation at 1,500 × *g* for 15 min. Serum, 100 µL/well, was aliquoted to duplicate wells of a dark 96 well plate (Greiner Bio-one, Monroe, NC). The degree of fluorescence was enumerated using an excitation wavelength of 485 nm and an emission wavelength of 528 nm using a ICTOR3 Multilabel Plate Reader (Perkin Elmer, Waltham, MA). The fluorescence concentrations were expressed as relative to the SCC group.

5.3.5. Ileal morphology and goblet cell counting

Alcian blue/period acid-schiff (AB/PAS) staining was used to assess villus height (VH), crypts depth (CD), VH:CD ratio as well as, to enumerate goblet cell number per 100 µm

VH and 100 μm CD in the ileum. The ileum samples were cut by blades and put it in cassettes. Each section was stained with alcian blue for 15 min and washed with distilled water. Samples were treated with periodic acid for 5 min and washed with distilled water. Subsequently, the samples were stained with Schiff's reagents for 10 min and washed with distilled water. The samples were counterstained in haematoxylin for 1 min and washed and dehydrated. The stained sections were viewed with a BZ microscope (BZ-X810; Keyence, Osaka, Japan). The villus height, crypt depth, and goblet cell numers of captured images (4×) were measured using ImageJ (National Institutes of Health, Bethesda, MD).

5.3.6. Activities of ileal brush border digestive enzymes and serum alkaline phosphatase

Mid-ileal tissue samples, 100 mg, were homogenized in 2 mL PBS using a beads beater (Biospec Products, Bartlesville, OK). The supernatant of homogenized samples after the centrifugation at 4 °C and 12,000 × *g* for 15 min was collected to analyze protein content using Pierce BCA Protein Assay Kits (Thermo Fisher Scientific, Waltham, MA) after 10-fold sample dilution. Activities of maltase and sucrase were determined according to Fan et al. (2004). Activities of alanine-aminopeptidase (APN) were analyzed according to Maroux et al. (1973). Activities of serum alkaline phosphatase and intestinal alkaline phosphatase were determined according to Lackeyram et al. (2010). Lipase activities were determined according to Elgharbawy et al. (2018). The activities of digestive enzymes were shown as values per mg protein.

5.3.7. Total antioxidant capacity, total glutathione, oxidized glutathione, and activities of superoxide dismutase measurement

Liver samples (100 mg each) were homogenized in 1 mL of the designated solution using a beads beater (Biospec Products, Bartlesville, OK) for each analysis including total antioxidant capacity (TAC), glutathione (GSH) and oxidized GSG (GSSG), and superoxide dismutase (SOD). Total antioxidant capacity (TAC) of the liver tissues was measured using a commercial kit (QuantiCromAntioxidant Assay Kit; DTAC-100, BioAssay Systems, Hayward, CA) after 2-time sample dilution. Total GSH and GSSG (D 21 liver tissues) were analyzed after diluting 20 times and 2 times, respectively, by using Caymans GSH assay kits (Cayman Chemical, Ann Arbor, MI). The activities of SOD (D 21 liver tissues) were analyzed using Caymans GSH assay kits (Cayman Chemical, Ann Arbor, MI) after 400 times dilution. Aliquots of the supernatants were collected for analysis of protein content using Pierce BCA Protein Assay Kits (Thermo Fisher Scientific, Waltham, MA) with 1:9 dilution. The TAC, GSH and GSSG concentrations, and SOD activities were expressed as values per mg protein.

5.3.8. RNA extraction and real-time reverse transcription (RT)-PCR analysis

Approximately, 100 mg of the mid-ileal samples were homogenized in QIAzol lysis reagents (Qiagen, Valencia, CA) using a bead beater (Biospec Products, Bartlesville, OK), and RNA was extracted according to the manufacturer's procedure. RNA quantity and purity were checked using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). One μg RNA was used to produce the first-strand cDNA using high-capacity cDNA synthesis kits (Applied Biosystems, Foster City, CA). Primers used in the study are shown in Table 5.2. Real-time reverse transcription PCR (RT-PCR) was performed using SYBR Green Master Mix

with a Step One thermocycler (Applied Biosystems, Foster City, CA). The final PCR volume (10 μL) contained 5 μL of SYBR Green Master Mix, 1.5 μL of cDNA, 0.5 μL of forward and reverse primers (10 μM), and 2.5 μL of water. Thermal cycle conditions for all reactions were as follows: 95 °C denature for 10 min, 40 cycles at 95 °C for 15 s and 60 °C for 1 min, 95 °C for 15 s, 60 °C for 1 min and 95 °C for 15 s. Several PCR products from each gene were stained with 6 × DNA loading dye (Thermo Fisher Scientific, Waltham, MA), electrophoresed on a 3% agarose gel in a Tris-acetate-EDTA buffer, and visualized by adding ethidium bromide, and the melting curve of each gene was then checked to confirm the specificity of each PCR product. Beta-actin and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used as the housekeeping genes (reference genes). The target mRNA abundance was normalized with geometric means of housekeeping genes (Vandesompele et al., 2002). Relative mRNA abundance was determined by using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The negative control, containing no cDNA, was included in each run, and each sample was run in duplicate.

Table 5.2. Primers used in the study

Genes[1]	Sequence, 5′ to 3′	Amplicon
Beta actin	F: CAACACAGTGCTGTCTGGTGGTA R: ATCGTACTCCTGCTTGCTGATCC	205
GAPDH	F: GCTAAGGCTGTGGGGAAAGT R: TCAGCAGCAGCCTTCACTAC	161
TLR2	F: CGGTGGAAAGGGAGAAAG R: CTTGCCACATCAGCTTCATT	103
TLR4	F: CCTGGACTTGGACCTCAGTT R: TTGTATGGATGTGGCACCTT	110
TLR5	F: CGTTAGTGAGAATGGCTGGA R: TGAGCCCATTGTATGAGAGC	106
NFκB	F: GAAGGAATCGTACCGGGAACA R: CTCAGAGGGCCTTGTGACAGTAA	131
IL1b	F: TGCCTGCAGAAGAAGCCTCG R: GACGGGCTCAAAAACCTCCT	204
IL2	F: CGTAAGTGGATGGTTTTCCTCT R: GGCTAAAGCTCACCTGGGTC	161
IL6	F: ATAAATCCCGATGAAGTGG R: CTCACGGTCTTCTCCATAAA	146
IL10	F: CTGTCACCGCTTCTTCACC R: CCCGTTCTCATCCATCTTCT	85
CD36	F: CTGGGAAGGTTACTGCGATT R: GGATCTGCAAATGTCAGAGG	109
CD74	F: GAAATCAGACCCCAGGAAGA R: GGTCTCAAAATCCTGCCAGT	109
CD80	F: ACCCTCTTTGTTACCGCTGA R: GTTTGGGAAAACCTCCATGA	118
SGLT1	F: GCC ATG GCC AGG GCT TA R: CAATAACCTGATCTGTGCACCAGTA	66
PepT1	F: CCCCTGAGGAGGATCCTT R: CAAAAGAGCAGCAACGA	66
EAAT3	F: TGCTGCTTTGGATTCCAGTGT R: AGCAATGACTGTAGTGCAGAAGTAATATATG	79
B0AT1	F: GGGTTTTGTGTTGGCTTAGGAA R: TCCATGGCTCTGGCAGAGAT	60
MUC2	F: ATGCGATGTTAACACAGGACTC R: GTGGAGCACAGCAGACTTTG	110
IAP	F: CTTCCTCGGAGATGGATTTG R: AGAGCCACATAGGGGAAAGA	123

[1] GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; TLR, toll like receptor; NFκB, nuclear factor kappa-light-chain-enhancer of activated B cells; IL, interleukin; CD, cluster of

differentiation; SGLT1, sodium glucose transporter 1; PepT1, peptide transporter 1; EAAT3, excitatory amino acid transporter 3; B0AT1, Sodium-dependent neutral amino acid transporter 1; MUC2, mucin 2; IAP, intestinal alkaline phosphatase

5.3.9. Peripheral blood leukocyte enrichment

On D18, blood samples (10 mL) were collected from one bird per pen after euthanizing via cervical dislocation. The blood samples were collected using 18 g needles (1.5 inches; World Precision Instruments, Inc., Sarasota, FL) with syringes and transferred into the Na-heparin 10 mL glass tubes (Grainer Bio-One, Kremsmuenster, Austria). To collect buffy coat, blood samples were centrifuged at 20 × *g*, 23 °C for 10 min with the acceleration and brake set to 0. The buffy coat was carefully collected by gently stirring the top plasma layer using an electronic pipette equipped with a sterile 9" glass pipette and transferred to a sterile 15 mL conical tube (Thermo Fisher Scientific, Waltham, MA) to create a liquid funnel. This technique lifted the leukocyte fraction into the plasma layer to facilitate collection. The samples were centrifuged at 250 × *g*, 23 °C for 10 min to collect leukocytes, and the supernatant was discarded, and sterile PBS was added. Leukocyte cell recovery and viability were measured on a Nexcelom Cell Counter (Nexcelom Bioscience, Lawrence, MA). The cell gates were set between 5 to 30 µm, and viability was measured via trypan blue exclusion. For the cell phenotyping and proliferation assays, the cells were diluted to 4.0×10^6/mL in complete RPMI medium (Gibco, Grand Island, NY) media containing 10% FBS (heat inactivated, USDA Approved; Gibco, Grand Island, NY), 2 mM L-glutamine (Gibco, Grand Island, NY), 50 IU/mL penicillin (HyClone Lab, Inc.; Logan, UT), and 50 µg/mL streptomycin (HyClone Lab, Inc.; Logan, UT).

5.3.10 Peripheral T cell phenotyping using flow cytometry

Flow cytometry analysis was conducted according to Krunkosky et al. (2020) with some modifications. A cell suspension of 100 µL (4.0×10^5) was aliquoted into wells followed

by 100 µL /well of CD4 (Mouse Anti-Chicken CD4-PE), CD8 (Mouse Anti-Chicken CD8a-FITC), and CD44 (Mouse Anti-Chicken CD44-PE) antibodies purchased from Southern Biotech (Birmingham, AL). The final antibody concentrations per reaction of PE anti-CD4 (clone CT-4), FITC anti-CD8 (clone CD8alpha), and PE anti-CD44 (clone CD44) were set to 0.5 µg/4.0 × 10^5 cells. After the addition of the conjugated antibodies, the samples were incubated for 30 min at 4 °C on an orbital mixer (New Brunswick Scientific, New Brunswick, NJ). The cells were then washed with 200 µL FACS-PBS buffer and centrifuged at 250 × *g*, 4 °C for 10 min. The supernatant was carefully aspirated from each well, and the 100 µL FACS-PBS buffer and 100 µL flow fixing solution (Thermo Fisher Scientific, Waltham, MA) were added. The stained cell suspension was then transferred to individual flow tubes. The stained cell suspensions were analyzed on a BD Accuri C6 Flow Cytometer (San Jose, CA). Gating was set according to a previously determined P1 gate (FSCA vs FSC-H) of enriched peripheral blood leukocytes (>90% lymphocytes) and the P2 gate (FSC-A vs SSCA). Gate setting is shown in Figure 5.1. A total of 10,000 events were obtained per sample in the lymphocyte gate. Values were reported as percentage of expression of gated leukocyte population.

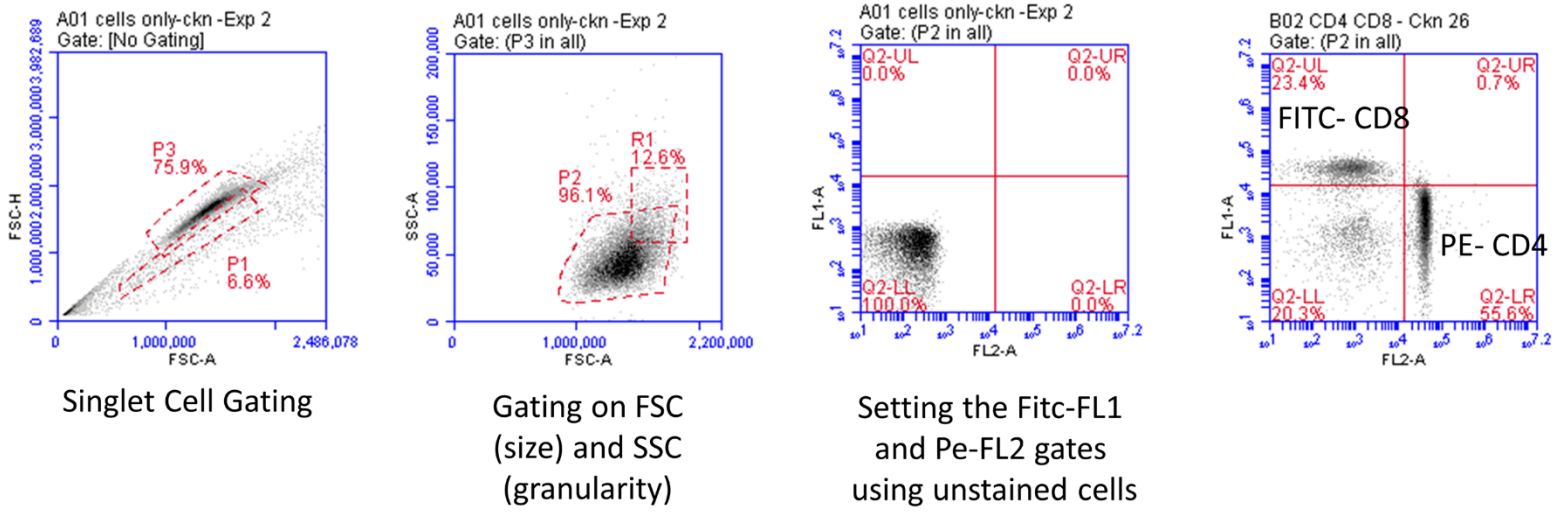

Figure 5.1. Gate setting for flow cytometry

5.3.11. Peripheral blood lymphocyte proliferation assay measure by alamar blue

Lymphocyte proliferation by using concanavalin A (Con A) was analyzed according to Ahmed et al. (1994) with modifications. Aliquots of cells, 100 μL/well (4.0×10^5) in complete RPMI 1640, were transferred in the sterile 96 wells of round bottom plates to triplicate wells containing 0.0, 0.1, 1, 10, and 20 μg/well of Con A. The plates were incubated for 48 h under humidified conditions with 95% O2 and 5% CO2 at 37 °C. After 48 h of culture, 20 μL/well of Alamar blue (Thermo Fisher Scientific, Waltham, MA) were added, and the plates were incubated an additional 24 h. After 72 h of culture, the plates were placed in a BioTek Synergy 4 plate reader (Biotek, Winooski, VT), and the absorbances at 570 and 600 nm were measured to assess cell proliferative response. The absorbance at 600 nm was subtracted from the absorbance at 570 nm. The slope was calculated as follows: average of differences between 570 nm and 600 nm / the four different amounts (0.0, 0.1, 1, 10, 20 μg per well) of Con A, and the slope was used as the value to represent peripheral blood lymphocyte proliferation by using Con A.

5.3.12. Peripheral blood leukocyte cytology

Cell suspensions, 20 μL (approximately 8×10^4 cells), were aliquoted into individual cytospin slide chambers containing 180 μL of Ca free/Mg free PBS (Fisher Scientific, Fair Lawn, NJ). Slide chambers were centrifuged at 34 × *g* for 3 min at 23°C using a 7150 Hematology Slide-Stainer Cytocentrifuge (Wescor, Logan, UT). Slides were then stained with Wright-Giemsa (Sigma-Aldrich, St. Louis, MO) using a 10 min full stain/10 min diluted stain protocol. Stained slides were evaluated on an Olympus CX43 compound light microscope (Olympus America Inc., Center Valley, PA) under 600× magnification. A total of 200

leukocytes across a minimum of 10 fields were enumerated to determine lymphocyte purity percentage. Examples of stained leukocytes are shown in Figure 5.2. Values were expressed as mean %.

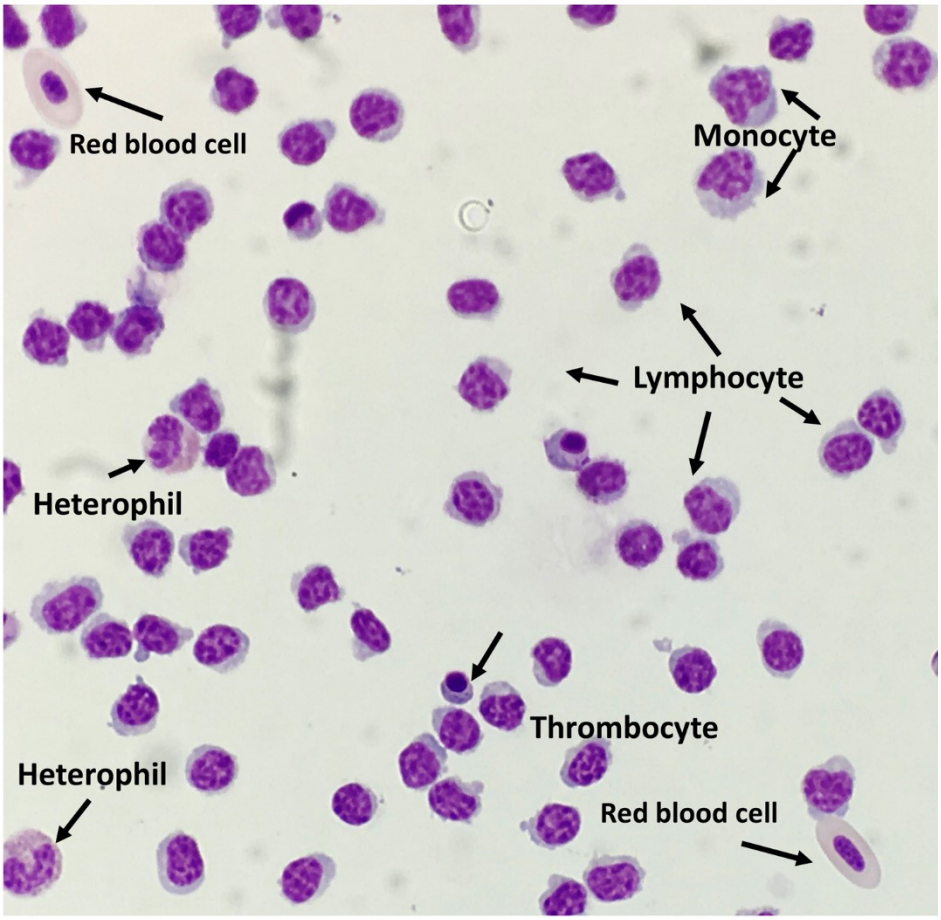

Figure 5.2. Different leukocytes in the peripheral blood

5.3.13. Statistical analyses

Statistical analyses were performed using SAS (version 9.4; SAS Inst. Inc., Cary, NC). Data normality was checked using proc univariate except for the results of presence of ST^{NR} in the spleen and liver. The presence of ST^{NR} in the spleen and liver was analyzed by chi-square analyses. The effects of ST^{NR} inoculation (SCC v. CC) were evaluated by the unpaired t-test. Challenged groups (CC, TA0.25, TA0.5, TA1, and TA2) were compared using PROC MIXED in a completely randomized design followed by the Tukey's comparison test. Orthogonal polynomial contrasts were utilized to evaluate the significance of linear or quadratic effects of different concentrations of TA within the treatments infected with ST^{NR}. Statistical significance was set at $P < 0.05$, and trends ($0.05 \leq P \leq 0.1$) were also presented.

5.4. RESULTS

5.4.1. Growth performance

Infection with ST^{NR} tended to reduce BW ($P = 0.096$) and ADG ($P = 0.098$) and significantly decreased ADFI ($P < 0.05$) on D 7 (Table 5.3.). Supplemental TA tended to linearly reduce BW ($P = 0.087$) and ADG ($P = 0.074$) in broilers infected with ST^{NR} on D 7. In the grower phase (D 7 to 21), ADG showed a linear increasing trend in TA supplemented groups ($P = 0.097$).

Table 5.3. Effects of supplemental tannic acid on the growth performance [body weight (BW), average daily gain (ADG), average daily feed intake (ADFI), and feed conversion ratio (FCR)] of broilers infected with *Salmonella* Typhimurium nalidixic acid resistant strain (ST^{NR}) in the starter phase (D 0 to 7), grower phase (D 7 to 21), and whole phase (D 0 to 21)

		ST^{NR}-challenged[2]							Polynomial contrast	
Items	SCC[1]	CC	TA0.25	TA0.5	TA1	TA2	SEM	*P* value	Linear	Quadratic
Initial BW, g	49.61	49.51	49.55	49.59	49.51	49.61	0.21	0.61		
Starter phase (D 0 to 7)										
BW, g	168.8*	162.9	163.8	159.7	159.4	156	8.56	0.471	0.087	0.850
ADG, g/d	16.22*	16.20	16.32	15.73	15.68	15.18	1.21	0.425	0.074	0.855
ADFI, g/d	19.48**	17.97	18.11	17.34	17.81	16.81	1.63	0.570	0.160	0.809
FCR, g/g	1.15	1.11	1.11	1.10	1.13	1.11	0.05	0.863	0.887	0.479
Grower phase (D 7 to 21)										
BW, g	543.6	491	525.24	538.35	540.72	541.65	61.58	0.513	0.215	0.268
ADG, g/d	27.20	23.47	25.91	27.17	27.34	27.61	4.03	0.312	0.097	0.204
ADFI, g/d	47.33	42.71	43.78	46.45	45.25	46.73	5.63	0.639	0.224	0.569
FCR, g/g	1.98	1.98	1.78	1.79	1.71	1.81	0.26	0.421	0.402	0.122
Whole phase (D 0 to 21)										
ADG, g/d	17.85	15.78	17.03	17.52	17.59	17.6	2.18	0.494	0.215	0.250
ADFI, g/d	38.05	34.47	35.22	36.75	36.1	36.76	4.04	0.796	0.345	0.575
FCR, g/g	1.7	1.69	1.55	1.56	1.52	1.57	0.18	0.477	0.423	0.149

SCC (sham-challenged control): broilers fed a basal diet and challenged with peptone water; CC (challenged control): broilers fed a basal diet and challenged with 0.5 mL of 1 × 10^8 CFU/mL of ST^{NR}; TA0.25 (tannic acid 250 mg/kg): CC + 250 mg/kg of tannic acid;

TA0.5 (tannic acid 500 mg/kg): CC + 500 mg/kg of tannic acid; TA1 (tannic acid 1,000 mg/kg): CC + 1,000 mg/kg of tannic acid; and TA2 (tannic acid 2,000 mg/kg): CC + 2,000 mg/kg of tannic acid;

[1] SCC vs CC (unpaired *t* test): * $0.05 < P < 0.10$, ** $P < 0.05$, *** $P < 0.01$

[2] ST^{NR} infected groups (CC, TA0.25, TA0.5, TA1, and TA2) were compared by PROC MIXED followed by the Tukey's multiple comparison test.

5.4.2. Colonization of ST^{NR} in the ceca, liver, and spleen

Colonization of ST^{NR} was measured in the ceca, liver, and spleen in broilers infected with ST^{NR} (Table 5.4.). On D 7, TA supplementation linearly ($P < 0.01$) and quadratically ($P < 0.05$) reduced ST^{NR} colonization in the ceca, and the TA1 and TA2 groups had significantly lower ST^{NR} colonization in the ceca compared to the CC group ($P < 0.05$). On D 14, supplemental TA significantly modulated ST^{NR} colonization in the liver ($P < 0.05$).

Table 5.4. Effects of supplemental tannic acid on the colonization of *Salmonella* Typhimurium nalidixic acid resistant strain (ST^{NR}) in the cecal content (log10), liver (infected birds per treatment), and spleen (infected birds per treatment) in broilers infected with ST^{NR} on D 4, 7, 14, and 21[1].

		ST^{NR}-challenged							Polynomial contrast	
Items	SCC	CC	TA0.25	TA0.5	TA1	TA2	SEM	*P* value	Linear	Quadratic
Cecal content[1]										
D 4		5.86	6.96	6.01	5.94	6.37	1.25	0.460	0.931	0.755
D 7		6.23[a]	4.8[ab]	5.11[ab]	2.59[b]	3.45[b]	1.66	0.002	0.002	0.018
D 14		3.12	0.79	3.44	3.01	1.20	2.21	0.101	0.314	0.294
D 21		1.30	2.13	2.60	2.78	1.96	2.26	0.762	0.736	0.208
Liver[2]										
D 4		3/7	5/7	5/7	3/7	3/7		0.598		
D 7	Not	4/7	6/7	2/7	4/7	1/7		0.069		
D 14	detected	4/7	0/7	0/7	4/7	1/7		0.013		
D 21		3/7	5/7	5/7	3/7	3/7		0.598		
Spleen[3]										
D 4		2/7	2/7	3/7	2/7	1/7		0.844		
D 7		2/7	1/7	1/7	1/7	1/7		0.938		
D 14		3/7	0/7	0/7	1/7	1/7		0.136		
D 21		2/7	2/7	3/7	2/7	1/7		0.844		

SCC (sham-challenged control): broilers fed a basal diet and challenged with peptone water; CC (challenged control): broilers fed a basal diet and challenged with 0.5 mL of 1 × 10^8 CFU/mL of ST^{NR}; TA0.25 (tannic acid 250 mg/kg): CC + 250 mg/kg of tannic acid; TA0.5 (tannic acid 500 mg/kg): CC + 500 mg/kg of tannic acid; TA1 (tannic acid 1,000 mg/kg): CC + 1,000 mg/kg of tannic acid; and TA2 (tannic acid 2,000 mg/kg): CC + 2,000 mg/kg of tannic acid;

[1] ST^{NR} infected groups (CC, TA0.25, TA0.5, TA1, and TA2) were compared by PROC MIXED followed by the Tukey's multiple comparison test for cecal samples. Different letters in the same row means significant differences ($P < 0.05$) among treatments.

[2,3] For the presence of ST^{NR} in the liver and spleen, ST^{NR} infected groups (CC, TA0.25, TA0.5, TA1, and TA2) were compared by chi-square test for independence

5.4.3. FITC-D4 flux and serum endotoxin concentrations

The FITC-D4 flux concentrations were analyzed on D 14 and 21, and serum endotoxin concentrations were analyzed on D 7 and 21 (Table 5.5). On D 14, infection with ST^{NR} significantly increased FITC-D4 flux, and the TA0.5 group had the higher FITC-D4 flux compared to the CC and TA2 ($p < 0.05$). On D 21, ST^{NR} infection significantly increased serum endotoxin concentrations ($P < 0.05$), and serum endotoxin concentrations were quadratically decreased by the TA supplementation ($P < 0.05$).

5.4.4. Ileal morphology and number of the goblet cells in the ileum

Ileal morphology and the number of goblet cells in the ileum were evaluated on D 7 and 21 (Table 5.6.). On D 7, the ST^{NR} infection tended to decrease ileal VH ($P = 0.084$) and VH:CD ($P = 0.082$). No significant differences in ileal morphology and number of goblet cells in the ileum were observed across the treatment groups. On D 21, ST^{NR} infection significantly reduced ileal VH and VH:CD. Supplemental TA quadratically increased ileal VH ($P < 0.05$), and the TA0.25 group had higher ileal VH compared to the CC group ($P < 0.05$). Goblet cells per 100 μm VH were also linearly increased due to TA supplementation ($P < 0.05$), and the TA2 group had significantly a higher numbers of goblet cells per 100 μm VH compared to the CC group ($P < 0.05$).

Table 5.5. Effects of supplemental tannic acid on the gut permeability [flux of fluorescein isothiocyanate–dextran (FITC-D4; average mol wt: 4000); D 14 and 21] and serum endotoxin concentrations (EU/mL; D 7 and 21) in broilers infected with *Salmonella* Typhimurium nalidixic acid resistant strain (ST^{NR}).

		ST^{NR}-challenged[2]							Polynomial contrast	
Items	SCC[1]	CC	TA0.25	TA0.5	TA1	TA2	SEM	*P* value	Linear	Quadratic
FITC-D4 flux relative to the SCC group										
D 14	1**	1.54^{b}	1.89^{ab}	2.33^{a}	1.82^{ab}	1.58^{b}	0.46	0.022	0.348	0.022
D 21	1	1.1	1.03	1.04	1.13	1.14	0.2	0.768	0.377	0.841
Serum endotoxin										
D 7	3.43	3.56	3.43	3.98	3.82	3.62	0.53	0.352	0.781	0.168
D 21	2.97***	3.28	3.11	3.12	3.02	3.34	0.25	0.136	0.385	0.018

SCC (sham-challenged control): broilers fed a basal diet and challenged with peptone water; CC (challenged control): broilers fed a basal diet and challenged with 0.5 mL of 1 × 10^8 CFU/mL of ST^{NR}; TA0.25 (tannic acid 250 mg/kg): CC + 250 mg/kg of tannic acid; TA0.5 (tannic acid 500 mg/kg): CC + 500 mg/kg of tannic acid; TA1 (tannic acid 1,000 mg/kg): CC + 1,000 mg/kg of tannic acid; and TA2 (tannic acid 2,000 mg/kg): CC + 2,000 mg/kg of tannic acid;

[1] SCC v. CC (unpaired *t* test): * $0.05 < P < 0.10$, ** $P < 0.05$, *** $P < 0.01$

[2] ST^{NR} infected groups (CC, TA0.25, TA0.5, TA1, and TA2) were compared by PROC MIXED followed by the Tukey's multiple comparison test. Different letters in the same row means significant differences ($P < 0.05$) among treatments.

Table 5.6. Effects of supplemental tannic acid on the ileal morphology [villus height (VH, μm), crypts depth (CD, μm), and VH:CD] and number of goblet cells per 100 μm VH and CD in the ileum of broilers infected with *Salmonella* Typhimurium nalidixic acid resistant strain (ST^{NR}) on D 7 and 21

		ST^{NR}-challenged[2]							Polynomial contrast	
Items	SCC[1]	CC	TA0.25	TA0.5	TA1	TA2	SEM	*P* value	Linear	Quadratic
D 7										
VH	560.1*	495.3	488.1	503.2	497.8	535.2	54.78	0.545	0.787	0.916
CD	132.1	135.3	124.5	131.4	138.6	134.2	16.87	0.601	0.425	0.270
VH:CD	4.34*	3.78	4.04	3.96	3.73	4.17	0.59	0.629	0.712	0.343
Goblet cells per 100 μm VH	9.45	9.78	9.41	9.30	10.30	8.87	1.12	0.200	0.355	0.124
Goblet cells per 100 μm CD	9.92	10.30	11.44	10.39	10.72	10.54	1.12	0.349	0.905	0.665
D 21										
VH	581.5**	454.1^{b}	568.9^{a}	506.9^{ab}	484.2^{ab}	491.9^{ab}	65.41	0.036	0.825	0.037
CD	113.9	112.6	127.1	116.3	115.0	121.9	16.49	0.486	0.710	0.385
VH:CD	5.18**	4.21	4.57	4.44	4.29	4.14	0.53	0.565	0.945	0.289
Goblet cells per 100 μm VH	11.7	11.14^{b}	9.70^{b}	11.69^{ab}	11.91^{ab}	13.54^{a}	1.47	0.001	0.052	0.498
Goblet cells per 100 μm CD	12.78	12.21	11.62	12.17	12.71	12.44	1.28	0.597	0.268	0.386

SCC (sham-challenged control): broilers fed a basal diet and challenged with peptone water; CC (challenged control): broilers fed a basal diet and challenged with 0.5 mL of 1 × 10^8 CFU/mL of ST^{NR}; TA0.25 (tannic acid 250 mg/kg): CC + 250 mg/kg of tannic acid; TA0.5 (tannic acid 500 mg/kg): CC + 500 mg/kg of tannic acid; TA1 (tannic acid 1,000 mg/kg): CC + 1,000 mg/kg of tannic acid; and TA2 (tannic acid 2,000 mg/kg): CC + 2,000 mg/kg of tannic acid;

[1] SCC v. CC (unpaired *t* test): * $0.05 < P < 0.10$, ** $P < 0.05$, *** $P < 0.01$

[2] ST^{NR} infected groups (CC, TA0.25, TA0.5, TA1, and TA2) were compared by PROC MIXED followed by the Tukey's multiple comparison test. Different letters in the same row means significant differences ($P < 0.05$) among treatments.

5.4.5. Activities of ileal brush border digestive enzymes and serum alkaline phosphatase

Activities of ileal brush border digestive enzymes and serum alkaline phosphatase were evaluated on D 7 and 21 (Table 5.7.). On D 7, aminopeptidase activities were significantly increased, and lipase activities were significantly decreased in ST^{NR} infected broilers compared to the SCC group. The TA fed broilers had linearly decreased maltase activities ($P < 0.05$), with the TA0.5 group having significantly higher maltase activities compared to the TA2 group ($P < 0.05$). The TA supplementation tended to enhance lipase activities ($P = 0.055$). On D 21, activities of serum alkaline phosphate were quadratically increased due to TA supplementation in broilers infected with ST^{NR} ($P < 0.05$). However, no differences were observed in the ileal brush border digestive enzymes on D 21 ($P > 0.1$).

5.4.6. Total antioxidant capacity, total glutathione, oxidized glutathione, and activities of superoxide dismutase

The total antioxidant capacity (TAC), total glutathione (GSH), oxidized GSH (GSSG), and superoxide dismutase (SOD) were evaluated in the ileum and liver on D 7 and 21 (Table 5.8.). No differences were observed across the treatments in the ileal TAC on D 7 and 21 ($P > 0.1$). Liver GSH concentrations and SOD activities were not different among the treatments on D 7 and 21.

Table 5.7. Effects of supplemental tannic acid on the activities of ileal brush border digestive enzymes [maltase (nmol glucose released/mg protein/min), sucrase (nmol glucose released/mg protein/min), aminopeptidase (nmol p-nitroaniline liberated/mg protein/min), lipase (mmol p-nitrophenyl phosphate liberated/mg protein/min), intestinal alkaline phosphatase (µmol p-nitrophenol liberated/mg protein/min), and serum alkaline phosphatase (µmol p-nitrophenol liberated/mL serum/min)] of broilers infected with *Salmonella* Typhimurium nalidixic acid resistant strain (ST^{NR}) on D 7 and 21

		ST^{NR}-challenged[2]							Polynomial contrast	
Items	SCC[1]	CC	TA0.25	TA0.5	TA1	TA2	SEM	*P* value	Linear	Quadratic
D 7										
Sucrase	0.81	1.30	1.11	1.42	1.08	1.76	0.69	0.359	0.169	0.276
Maltase	94.48	150.84^{ab}	109.35^{ab}	178.41^{a}	110.76$^{a}_{b}$	78.08^{b}	62.33	0.045	0.026	0.555
Aminopeptidase	38.90**	44.52	52.59	50.77	44.46	53.61	14.61	0.639	0.515	0.731
Lipase	0.92**	0.58	0.62	0.67	0.54	0.85	0.23	0.141	0.055	0.231
Intestinal alkaline phosphatase	0.15	0.18	0.22	0.21	0.18	0.29	0.21	0.482	0.148	0.475
Serum alkaline phosphatase	1.57	1.75	1.98	1.76	1.46	1.69	0.56	0.607	0.465	0.459
D 21										
Sucrase	0.90	0.53	1.24	0.45	1.38	0.77	1	0.340	0.780	0.306
Maltase	176.12	127.71	148.29	118.96	268.75	170.79	196.48	0.631	0.504	0.360
Aminopeptidase	44.80	52.40	57.86	45.82	73.87	57.90	34.23	0.640	0.596	0.485
Lipase	1.34	1.62	2.57	1.67	2.08	2.17	2.09	0.917	0.805	0.943

Intestinal alkaline phosphatase	0.26	0.34	0.54	0.29	0.65	0.38	0.39	0.415	0.884	0.294
Serum alkaline phosphatase	1.97	1.83	2.17	2.01	2.29	1.88	0.42	0.233	0.906	0.047

SCC (sham-challenged control): broilers fed a basal diet and challenged with peptone water; CC (challenged control): broilers fed a basal diet and challenged with 0.5 mL of 1 × 10^8 CFU/mL of ST^{NR}; TA0.25 (tannic acid 250 mg/kg): CC + 250 mg/kg of tannic acid; TA0.5 (tannic acid 500 mg/kg): CC + 500 mg/kg of tannic acid; TA1 (tannic acid 1,000 mg/kg): CC + 1,000 mg/kg of tannic acid; and TA2 (tannic acid 2,000 mg/kg): CC + 2,000 mg/kg of tannic acid;

[1] SCC v. CC (unpaired *t* test): * $0.05 < P < 0.10$, ** $P < 0.05$, *** $P < 0.01$

[2] ST^{NR} infected groups (CC, TA0.25, TA0.5, TA1, and TA2) were compared by PROC MIXED followed by the Tukey's multiple comparison test. Different letters in the same row means significant differences ($P < 0.05$) among treatments.

Table 5.8. Effects of supplemental tannic acid on the total antioxidant capacity (TAC; μM Trolox Equivalents/mg protein; D 7 and 21) of the ileum and TAC, activities of superoxide dismutase (SOD; U/mg protein), and concentrations of glutathione (GSH; μM/mg protein), oxidized GSH (GSSG; μM/mg protein), reduced GSH (μM/mg protein), and reduced GSH:GSSG of the liver in D 21 broilers infected with *Salmonella* Typhimurium nalidixic acid resistant strain (ST^{NR}).

		ST^{NR}-challenged[2]							Polynomial contrast	
Items	SCC[1]	CC	TA0.25	TA0.5	TA1	TA2	SEM	*P* value	Linear	Quadratic
Ileum TAC										
D 7	56.08	57.38	56.31	57.28	63.06	58.63	6.59	0.356	0.376	0.209
D 21	68.41	60.22	74.35	65.48	61.87	61.67	14.14	0.354	0.471	0.745
D 21 Liver										
TAC	100	104.65	105.32	103.74	108.10	101.88	9.66	0.814	0.643	0.411
SOD	19.08	21.23	22.59	25.64	21.55	20.93	6.14	0.608	0.543	0.456
GSH	36.13	35.96	30.96	30.51	34.60	29.67	5.45	0.180	0.202	0.995
GSSG	1.28	1.62	1.27	1.34	1.68	1.48	0.46	0.418	0.766	0.948
Reduced GSH[3]	28.14	32.71	28.41	27.84	31.23	26.71	4.87	0.172	0.140	0.995
Reduced GSH:GSSG	23.36	20.24	22.62	21.27	20.04	19.11	4.27	0.608	0.272	0.725

SCC (sham-challenged control): broilers fed a basal diet and challenged with peptone water; CC (challenged control): broilers fed a basal diet and challenged with 0.5 mL of 1 × 10^8 CFU/mL of ST^{NR}; TA0.25 (tannic acid 250 mg/kg): CC + 250 mg/kg of tannic acid;

TA0.5 (tannic acid 500 mg/kg): CC + 500 mg/kg of tannic acid; TA1 (tannic acid 1,000 mg/kg): CC + 1,000 mg/kg of tannic acid; and TA2 (tannic acid 2,000 mg/kg): CC + 2,000 mg/kg of tannic acid;

[1] SCC v. CC (unpaired *t* test): * $0.05 < P < 0.10$, ** $P < 0.05$, *** $P < 0.01$

[2] ST^{NR} infected groups (CC, TA0.25, TA0.5, TA1, and TA2) were compared by PROC MIXED followed by the Tukey's multiple comparison test. Different letters in the same row means significant differences ($P < 0.05$) among treatments.

[3] Reduced GSH = total GSH – 2 × GSSG

5.4.7. Relative mRNA expression of genes related to the immune system and nutrient transporters

The mRNA expression of genes related to immunity and nutrient transporters in the ileum was evaluated on D 7 and 21 (Table 5.9.). On D 7, ST^{NR} infection significantly reduced mRNA expression of toll like receptor 2 (TLR2; $P < 0.05$) and increased mRNA expression of nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB; $P = 0.067$). The ST^{NR} infection increased mRNA expression of mucin 2 (MUC2; $P = 0.061$). The TA supplementation tended to linearly decrease mRNA expression of excitatory amino acid transporter 3 (EAAT3; $P = 0.075$). On D 21, the CC group had significantly decreased mRNA expression of TLR2 ($P < 0.01$) and TLR4 ($P < 0.05$) compared to the SCC group. The mRNA expression of interleukin 6 (IL6) was quadratically decreased in TA supplemented birds ($P < 0.05$). The mRNA expression of cluster of differentiation 36 (CD36), CD74, and sodium glucose transporter 1 (SGLT1) was significantly decreased in the CC group compared to the SCC group ($P < 0.05$). The TA supplementation trended toward a linear increase in mRNA expression of peptide transporter 1 (PepT1; $P = 0.095$). The ST^{NR} infected birds had significantly decreased mRNA expression of EAAT3 and sodium-dependent neutral amino acid transporter (B0AT1), while supplementation with TA yielded a linear increase in EAAT3 expression ($P < 0.05$). On D 21, ST^{NR} infection trended toward a decrease in mRNA expression of intestinal alkaline phosphatase ($P = 0.063$).

Table 5.9. Effects of supplemental tannic acid on the relative mRNA expression ($2^{-\Delta\Delta Ct}$) of genes related to immune system and nutrient transporters in the ileum of broilers infected with *Salmonella* Typhimurium nalidixic acid resistant strain (ST^{NR}) on D 7 and 21[1]

		ST^{NR}-challenged[3]							Polynomial contrast	
Items	SCC[2]	CC	TA0.25	TA0.5	TA1	TA2	SEM	*P* value	Linear	Quadratic
D 7										
TLR2	1.04**	0.69	0.68	0.77	0.73	0.70	0.27	0.968	0.932	0.624
TLR4	1.08	1.15	0.99	1.12	1.04	1.05	0.36	0.924	0.777	0.823
TLR5	1.17	1.18	1.06	1.12	0.81	0.96	0.48	0.625	0.303	0.354
NFκB	1.04*	1.46	1.28	1.33	1.06	1.18	0.35	0.276	0.123	0.167
IL1b	1.14	1.19	1.26	1.28	0.73	1.23	0.58	0.365	0.722	0.193
IL2	2	1.91	1.03	0.88	1.77	0.77	1.67	0.593	0.445	0.937
IL6	1.27	1.14	0.57	0.59	0.79	0.51	0.84	0.635	0.376	0.683
IL10	1.14	0.97	1.06	1.71	1.08	0.66	0.92	0.336	0.302	0.215
CD36	1.12	0.87	0.37	0.90	0.65	0.76	0.62	0.517	0.885	0.752
CD74	1.16	1.12	1.08	1.15	1.03	0.98	0.41	0.930	0.440	0.950
CD80	1.03	1.25	1.25	1.25	1.25	1.25	0.56	0.953	0.589	0.572
SGLT1	1.01	1.07	1.22	1.22	1.00	1.50	0.57	0.533	0.235	0.427
PepT1	1.05	0.86	0.93	0.74	0.82	0.98	0.34	0.706	0.518	0.365
EAAT3	1.22	1.44	1.24	1.80	0.78	0.70	0.98	0.214	0.075	0.991
MUC2	1.11*	1.54	1.87	1.71	1.58	2.00	0.6	0.578	0.304	0.611
B0AT1	1.08	1.40	1.39	1.30	0.99	1.19	0.39	0.294	0.166	0.160

IAP	1.05	0.93	1.16	1.43	1.29	1.44	0.44	0.188	0.072	0.269
D 21										
TLR2	1.02***	0.60	0.75	0.83	0.63	0.82	0.49	0.849	0.604	0.983
TLR4	1.01**	0.70	0.68	0.91	0.64	0.90	0.32	0.364	0.325	0.688
TLR5	1.08	1.64	0.85	1.35	1.22	1.28	0.86	0.601	0.869	0.569
NFκB	1.01	0.99	1.09	1.18	1.16	1.28	0.36	0.656	0.169	0.698
IL1b	1.06	0.78	1.15	1.15	1.00	1.30	0.58	0.523	0.223	0.863
IL2	1.23	1.46	0.57	0.57	0.89	0.67	1	0.429	0.444	0.403
IL6	1.01	1.55	0.86	0.98	0.82	1.20	0.73	0.337	0.750	0.081
IL10	1.57	1.18	1.47	1.45	1.11	1.08	0.86	0.852	0.488	0.828
CD36	1.17**	0.47	0.44	0.84	0.44	0.51	0.37	0.298	0.905	0.549
CD74	1.03**	0.74	0.80	0.89	0.79	1.04	0.33	0.485	0.118	0.760
CD80	1.13	0.91	1.24	1.13	0.94	0.90	0.56	0.731	0.519	0.693
SGLT1	1.09**	0.53	0.83	0.88	0.65	0.83	0.34	0.285	0.416	0.633
PepT1	1.24	1.01	0.78	1.55	1.15	1.65	0.79	0.227	0.095	0.956
MUC2	1.48	1.14	1.42	1.23	1.04	1.46	0.57	0.606	0.545	0.419
EAAT3	1.07**	0.65	0.67	0.87	0.73	1.18	0.48	0.237	0.039	0.628
B0AT1	1.04**	0.68	0.67	0.97	0.75	1.12	0.51	0.378	0.101	0.831
IAP	1.05*	0.69	0.99	1.10	0.78	1.12	0.4	0.202	0.225	0.955

SCC (sham-challenged control): broilers fed a basal diet and challenged with peptone water; CC (challenged control): broilers fed a basal diet and challenged with 0.5 mL of 1 × 10^8 CFU/mL of ST^{NR}; TA0.25 (tannic acid 250 mg/kg): CC + 250 mg/kg of tannic acid;

TA0.5 (tannic acid 500 mg/kg): CC + 500 mg/kg of tannic acid; TA1 (tannic acid 1,000 mg/kg): CC + 1,000 mg/kg of tannic acid; and TA2 (tannic acid 2,000 mg/kg): CC + 2,000 mg/kg of tannic acid;

[1] GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; TLR, toll like receptor; NFκB, nuclear factor kappa-light-chain-enhancer of activated B cells; IL, interleukin; CD, cluster of differentiation; SGLT1, sodium glucose transporter 1; PepT1, peptide transporter 1; EAAT3, excitatory amino acid transporter 3; B0AT1, Sodium-dependent neutral amino acid transporter 1; MUC2, mucin 2; IAP, intestinal alkaline phosphatase

[2] SCC v. CC (unpaired *t* test): * $0.05 < P < 0.10$, ** $P < 0.05$, *** $P < 0.01$

[3] ST^{NR} infected groups (CC, TA0.25, TA0.5, TA1, and TA2) were compared by PROC MIXED followed by the Tukey's multiple comparison test.

5.4.8. Lymphocyte proliferation and immune cell phenotyping of enriched peripheral blood leukocytes

On D 18, peripheral blood leukocytes were enriched and assessed for T cell and pan-leukocyte phenotype as well as capacity to respond to a pan T cell mitogen, Con A (Table 5.10.). Average lymphocyte purity and viability were 87% and >89% across all treatment groups, respectively. Enriched peripheral blood leukocytes generated a Con A proliferation curve that was comparable across all treatment groups. However, no differences were observed among the treatments in the Con A proliferation rate ($P > 0.1$). When these same cells were phenotyped with pan-leukocyte cell marker $CD44^+$, the gated cell fraction averaged ≥ 95% leukocytes across all treatment groups, and the percentage of $CD4^+$ was comparable across all treatment groups. However, the percentage of $CD8^+$ cells was linearly significantly increased due to supplemental TA ($P < 0.01$), resulting in a decrease in the ratio of CD4:CD8 cells ($P < 0.05$).

5.4.9. Cytology and morphology of peripheral blood leukocytes

Cytologic assessment of the enriched peripheral blood leukocytes via cytospins revealed that the mean sample number across all treatments contained > 95.0% mononuclear cells with lymphocytes comprising ≥ 89.0% of the cells. As shown in Table 5.11., supplemental TA quadratically reduced lymphocyte abundance ($P < 0.05$), and the TA0.5 group had significantly lower lymphocytes compared to the CC groups ($P < 0.05$). Heterophils were the only other leukocyte enumerated from this enriched leukocyte fraction and ranged from 2 to 11%, except for two slide samples where chicken red blood cells averaged less than 4.0%.

When the results were compared across the challenged treatment groups, % of monocytes was linearly increased by supplemental TA ($P < 0.01$).

Table 5.10. Effects of supplemental tannic acid on the T cell proliferation by concanavalin A (Con A), proportion of CD4, CD8, and CD44 in the serum leukocytes of broilers infected with *Salmonella* Typhimurium nalidixic acid resistant strain (ST^{NR}) on D 18

		ST^{NR}-challenged[2]						Polynomial contrast	
Items	SCC[1]	CC	TA0.25	TA0.5	TA1	TA2	SEM	*P* value	Linear	Quadratic
T cell proliferation by Con A (Slope[3])	31.92	35.09	30.74	33.88	34.56	33.51	6.26	0.731	0.921	0.986
Leukocyte cell Phenotype										
$CD4^+$ (%)	54.86	52.99	50.96	53.88	50.86	51.01	5.92	0.852	0.540	0.925
$CD8^+$ (%)	22.90	22.69	21.94	20.02	27.09	28.01	4.95	0.033	0.006	0.935
CD4:CD8	2.51	2.66	2.39	2.85	1.92	1.92	0.8	0.162	0.042	0.708
$CD44^+$ (%)	95.70	95.46	92.07	91.78	95.24	95.00	2.93	0.072	0.269	0.355

SCC (sham-challenged control): broilers fed a basal diet and challenged with peptone water; CC (challenged control): broilers fed a basal diet and challenged with 0.5 mL of 1 × 10^8 CFU/mL of ST^{NR}; TA0.25 (tannic acid 250 mg/kg): CC + 250 mg/kg of tannic acid; TA0.5 (tannic acid 500 mg/kg): CC + 500 mg/kg of tannic acid; TA1 (tannic acid 1,000 mg/kg): CC + 1,000 mg/kg of tannic acid; and TA2 (tannic acid 2,000 mg/kg): CC + 2,000 mg/kg of tannic acid;

[1] SCC v. CC (unpaired *t* test): * $0.05 < P < 0.10$, ** $P < 0.05$, *** $P < 0.01$

[2] ST^{NR} infected groups (CC, TA0.25, TA0.5, TA1, and TA2) were compared by PROC MIXED followed by the Tukey's multiple comparison test.

[3] The slope was calculated as follows: average of differences between 570 nm and 600 nm / the four different amounts (0.0, 0.1, 1, 10, and 20 μg per well) of Con A

Table 5.11. Effects of supplemental tannic acid on enriched peripheral blood leukocytes (lymphocytes, monocytes, and heterophils) in broilers infected with *Salmonella* Typhimurium nalidixic acid resistant strain (ST^{NR}) on D 18.

		ST^{NR}-challenged[2]							Polynomial contrast	
Items	SCC[1]	TA0	TA0.25	TA0.5	TA1	TA2	SEM	*P* value	Linear	Quadratic
Lymphocytes	89.67	91.50[a]	92.50[a]	83.25[b]	87.67[ab]	88.75[ab]	3.3	0.015	0.301	0.037
Monocytes	4.33	5	3	6.5	7.67	8.5	2.52	0.062	0.016	0.523
Heterophils	0.67	1.25	1.75	2.5	2	1	1.08	0.364	0.443	0.111

SCC (sham-challenged control): broilers fed a basal diet and challenged with peptone water; CC (challenged control): broilers fed a basal diet and challenged with 0.5 mL of 1 × 10^8 CFU/mL of ST^{NR}; TA0.25 (tannic acid 250 mg/kg): CC + 250 mg/kg of tannic acid; TA0.5 (tannic acid 500 mg/kg): CC + 500 mg/kg of tannic acid; TA1 (tannic acid 1,000 mg/kg): CC + 1,000 mg/kg of tannic acid; and TA2 (tannic acid 2,000 mg/kg): CC + 2,000 mg/kg of tannic acid;

[1] SCC v. TA0 (unpaired *t* test): * $0.05 < P < 0.10$, ** $P < 0.05$, *** $P < 0.01$

[2] ST^{NR} infected groups (TA0, TA0.25, TA0.5, TA1, and TA2) were compared by PROC MIXED followed by the Tukey's multiple comparison test. Different letters in the same row means significant differences ($P < 0.05$) among treatments.

5.5. DISCUSSION

The purpose of the study was to investigate the effects of supplemental TA on growth performance, *Salmonella* colonization, serum endotoxin concentrations, ileal and hepatic antioxidant capacity, ileal morphology, gut barrier integrity, activities of ileal brush border digestive enzymes, serum alkaline phosphatase activities, and immunity in broilers infected with *S.* Typhimurium. The ST^{NR} infection model was implemented by administrating 0.5 mL of 10^8 ST^{NR} in peptone water on D 0, with ST^{NR} selected as the *S.* Typhimurium infection model in broilers. The NA is an antibiotic that has been commonly used in poultry production (Antunes et al., 2003). However, NA resistant strains of *S.* Typhimurium are adversely affecting broiler and human health (Campioni et al., 2017). There are already many antibiotic resistant strains of *Salmonella* spp. including tetracycline, ciprofloxacin, and ampicillin-resistant ones, and thus, novel strategies are needed to control the spread of these antibiotic resistant *Salmonella* spp. strains (Yuan and Guo, 2017). Furthermore, the NA resistant strains of *Salmonella* spp. have been frequently being used as a research model due to their easy management and analyses (Adhikari et al., 2018). Inoculation was conducted on D 0 because egg contamination from layers is the one of the common ways for ST^{NR} to infect young broilers (De Buck et al., 2004). On D 4 and 7, ST^{NR} concentrations in the ceca peaked, and after D 7, ST^{NR} concentrations decreased in the ceca of broilers.

Young broilers (newly hatched; D 0 to 7) are more susceptible to the *Salmonella* infection because their heterophils, one of the predominant immune cells in the young birds, have lower phagocytic ability, degranulation, and oxidative burst compared to the mature broilers (Alkie et al., 2019). In the current study, D 0 to 7 and D 7 to 21 was considered as

acute infection and chronic infection period, respectively. The ST^{NR} infection reduced BW and ADG on D 7 but did not affect growth performance on D 21. Decreased ADG on D 0 to 7 because of ST^{NR} infection would be mainly due to decreased ADFI in the current study. Reducing feed intake is one of the pathways pathogenic bacteria employ to decrease growth performance in broilers (Remus et al., 2014), which is consistent with our current study. Still, it is possible that ST^{NR}-induced fever as well as local and systemic stress may have contributed to a reduced appetite in these infected birds. Moreover, colonized ST^{NR} may have deprived the chicken host gut of nutrients (especially proteins) and beneficial microbes. Further, the ST^{NR} colonization can limit attachment sites for beneficial microbes (Nisbet et al., 1996).

The lower section of the gastrointestinal tract (GI) tract, where ST^{NR} mainly colonize, is the primary site to produce microbial metabolites (e.g., volatile fatty acids), which are important nutrients for gut development and energy homeostasis (Choi et al., 2021). Deficiency of nutrients and energy due to inadequate production of volatile fatty acids may lead to poor gut development, which can decrease feed intake in broilers (Allameh and Toghyani, 2019). Because ST^{NR} concentrations in the ceca of young broilers were higher in D 4 and 7 compared to those in D 14 and 21 in the current study, the effects of ST^{NR} colonization on growth performance would have been more severe in young broilers compared to the older broilers. In the current study, supplemental TA linearly decreased ADG on D 0 to 7, and supplemental TA linearly increased ADG on D 7 to 21 in ST^{NR} infected broilers. Young broilers may have been sensitive to dietary TA concentrations, which is consistent with a previous study (Chang and Fuller, 1964) reporting that tannins can severely limit nutrient digestion and show toxicity in young broilers compared to mature broilers. This would be

because young broilers have unmature gastrointestinal tract (e.g., lower enzyme activities, shorter passaging time, etc.) (Sell, 1996), which may lead to aggravate antinutritional effects (e.g., precipitation of nutrients and endogenous enzymes) of dietary TA in the gastrointestinal tract. Increased ADG in TA supplemented groups in the grower phase could be due to higher resistance to TA in the mature broilers as well as reduced cecal ST^{NR} concentrations on D 7 in the present study. Although supplemental TA negatively affected growth performance of broilers on D 7, TA supplementation in the grower phase can be beneficial to improve growth performance from D 7 to 21, as evident by an increased antimicrobial effect of TA against ST^{NR} on D 7.

The antimicrobial effects of TA against diverse pathogens have been demonstrated in previous *in vitro* studies (Graziani et al., 2006; Kim et al., 2010; Sivasankar et al., 2020). However, the antimicrobial effects of TA under *in vivo* conditions have not been reported, because TA cannot exhibit antimicrobial effects if it forms complexes with nutrients or reach the lower GI tract in sufficient concentrations, where many pathogens propagate (Van Parys et al., 2010). In the present study, supplemental TA linearly reduced cecal ST^{NR} load on D 7 and 1 and 2 g/kg TA supplementation significantly reduced cecal ST^{NR} load compared to the CC group but did not display antimicrobial effects on D 4. In other words, TA did not inhibit the initial colonization of ST^{NR}, but gradually reduced *Salmonella* colonization via exhibiting bacteriostatic and bactericidal effects against ST^{NR}. This observation implies that at least 1 g/kg of dietary TA should be supplemented in the feed to guarantee antimicrobial effects against ST^{NR} in broilers, and active forms (pure or partially degraded forms) of TA were able to seed the lower GI tract of the ST^{NR}-infected broilers. According to Choi and Kim (2020),

potential mode of actions of antimicrobial effects of TA are 1) increasing membrane permeability of bacteria by interacting with components of cell walls; 2) reducing activities of microbial enzymes via precipitation; and 3) depriving essential nutrients (e.g., iron) for ST^{NR} via precipitation.

Our current study reported that TA supplementation modulated colonization of ST^{NR} in the liver of broilers infected with ST^{NR}. S. Typhimurium that enters the blood stream mainly by invading enterocytes of the distal ileum or ceca to infect the internal organs of broilers (Lawhon et al., 2002). The invasion and colonization by ST^{NR} requires swarming motility and biofilm formation, which are controlled by cell communications (e.g., quorum sensing) (Kearns, 2010; Merino et al., 2019). Sivasankar et al. (2020) reported that TA showed anti-quorum sensing and anti-virulence activities under *in vitro* conditions, which could explain the reduced liver colonization of ST^{NR}, without affecting cecal colonization, that was observed on D 14 in the current study.

Gut barrier integrity, otherwise known as gut permeability, is a defensive mechanism against pathogenic bacteria and their toxins in the gut (Choi et al., 2020c). The FITC-D4 flux assay method is useful to measure gut permeability in broilers (Liu et al., 2021). In the present study, FITC-D4 flux was measured on D 14 and 21 rather than D 7 and 21 because the FITC-D4 method was previously optimized for only D 14 to 21 (Teng et al., 2020b). On D 14, ST^{NR} infection increased gut permeability, and supplemental TA quadratically increased gut permeability in broilers infected with ST^{NR}. A study reported that *Salmonella* infection caused disruption of tight junctions, which would suggest altered gut permeability (Wang et al., 2018), whereas other studies have reported that TA supplementation enhanced gut barrier integrity in

animals (Lopetuso et al., 2015; Scaldaferri et al., 2014; Yu et al., 2020). Thus, the observed aggravated gut barrier integrity in the TA fed birds is likely due to epithelial cell toxicity in the GI tract as the concentrations of TA were higher for these young broilers. Moreover, these gut permeability results suggest a possible TA contribution to the reduced growth performance.

The serum endotoxin concentrations (e.g., lipopolysaccharides; LPS) in broilers, produced by gram-negative bacteria during their growth, division, and death in the intestine, are affected by microbiota composition and activities, and gut permeability. Once endotoxins enter the blood circulation, they stimulate inflammatory reactions, which can lead to bird sepsis (Reisinger et al., 2020). In the current study, serum endotoxin concentrations were not modulated due to ST^{NR} infection, whereas it was confirmed that there were around log 6.23 CFU/g content of ST^{NR} in the ceca on D 7. However, there were only log 1.3 CFU/g content of ST^{NR} in the ceca on D 21, the serum endotoxin concentrations were increased on D 21 due to ST^{NR} infection. Other than *Salmonella* spp., diverse gram-negative bacteria such as *Escherichia coli*, *Pseudomonas hydrophila*, *Aeromonas hydrophila*, and *Vibrio damsel* produce endotoxins (El-Naggar et al., 2019). Moreover, no differences were observed in the gut permeability on D 21 in this study. Therefore, ST^{NR} was not the main intestinal microbe producing LPS in broilers in the present study. Continuous infection of ST^{NR} potentially altered the entire microbiota and their activities, which leads to increased serum endotoxin concentrations regardless of ST^{NR} concentrations in the ceca. In the current study, TA supplementation quadratically decreased serum endotoxin concentrations on D 21. This would suggest that TA favorably modified the gut microbiota and altered endotoxin production, which is supported in a previous study by Diaz Carrasco et al. (2018) reporting that TA

modulated the gut microbiome of broilers. Alkaline phosphatase has an important role detoxify LPS in the blood (Yang et al., 2012), and TA supplementation quadratically increased activities of serum alkaline phosphatase on D 21 in consistent with the serum endotoxin concentration. Potentially, activities of serum alkaline phosphatase was increased due to higher production of short chain fatty acids by cecal microbiota (Koyama and Ono, 1976) and/or developed gastrointestinal tract in broilers fed supplemental TA (Yang et al., 2012). Moreover, according to Osete-Alcaraz et al. (2020), TA interacts with polysaccharides, which would infer that TA can interact with LPS.

Intestinal morphology is an important indicator to reflect the capacity of nutrient digestion and absorption in broilers. The ileal section was selected for analyses for intestinal morphology and other gut health parameters because ST^{NR} inhabits in the lower intestine (ileum and ceca), and the ileum still has functions of nutrient digestion and absorption in broilers. Hence, we hypothesized that more differences would be observed in the ileal section in the current study. Results showed the ST^{NR} infection reduced ileal morphology in the ileum on D 7 and 21, which would be associated with induced inflammation due to ST^{NR} infection (Wu et al., 2018). The mRNA expression of TLR2 (an activator of the innate immune system) and NFκB (proinflammatory transcription factor related to inflammation, immune response, and cell apoptosis) were reduced due to ST^{NR} infection D 7 (Jin et al., 2020). While a statistical significance was not achieved (P = 0.123), TA supplementation linearly reduced mRNA expression of NFκB in the ileum, which supports that TA has an anti-inflammatory function (Huang et al., 2020). However, this did not lead to enhancement in ileal morphology on D 7. On D 21, ST^{NR} infection reduced mRNA expression of TLR2, TLR4, CD36, and CD74, which

are related to innate immunity (Liu et al., 2017a) and B cell activation (Gore et al., 2008). Huang et al. (2020) reported infection of *S.* Typhimurium inhibited the innate immune system and promoted cell apoptosis. On D 21, TA supplementation quadratically increased ileal morphology, and TA0.25 group significantly higher VH compared to the CC group. Supplemental TA quadratically decreased mRNA expression of IL6 (a proinflammatory cytokine) in the ileum on D 21 in the current study, which suggested that supplemental TA enhanced ileal morphology by showing anti-inflammatory effects. Moreover, mRNA expression of nutrient transporters including SGLT1 and EAAT3 was linearly enhanced by supplemental TA, which potentially explains linearly improved growth performance in the TA groups during the grower phase. Collectively, anti-inflammatory effects of TA may lead to improved ileal morphology and nutrient absorption of broilers infected with ST^{NR}.

Activities of ileal brush border enzymes are crucial indicators to reflect gut development and capacity of nutrient digestion in broilers. In the current study, ST^{NR} infection reduced lipase activities on D 7. Secreted lipase from pancreas is trapped in the mucus layer and digests lipids (Song et al., 2018), and lipase activities can be measured in the ileal tissue. Reduced lipase due to ST^{NR} infection in the current study potentially because ST^{NR} infection impaired pancreas function by inducing system inflammation (Wu et al., 2019). Increased lipase activities by supplemental TA might be the result of supplemental TA ameliorating impaired pancreas functions due to ST^{NR} infection in the current study. This is supported by Majumdar and Moudgal (1994) reported that TA showed beneficial effects on activities of pancreatin enzymes in broilers. In contrast, ST^{NR} infection increased aminopeptidase activities on D 7 in the current study. Enzyme activities are mainly affected by number of enzymes, and

Carroll et al. (2012) and Cheng et al. (2015) reported that aminopeptidase can provide colonization areas for bacteria. This possibly implies that aminopeptidase also can provide colonization areas for ST^{NR}, and ST^{NR} increased the number of aminopeptidases for their proliferation, which led to increased aminopeptidase activities in the present study. Supplemental TA reduced maltase activities on D 7 potentially because of forming a complex with maltase. This can partially support that supplemental TA can restrict nutrient digestion in the GI tract and show toxicity in young broilers infected with ST^{NR}.

Intestinal mucus, which is produced by mucin-secreting goblet cells in the epithelial layer, plays a crucial role as an innate defense against pathogens, supporting the colonization of commensal bacteria and facilitating nutrient digestion and absorption (Duangnumsawang et al., 2021). Increased mucus production can serve either as an indicator of bacterial infections or for enhanced gut health of broilers. In the current study, ST^{NR} infection increased mRNA expression of MUC2, which is the dominant mucin in the intestine (Yamashita and Melo, 2018). This result is in agreement with a previous study (Fasina et al., 2010) demonstrating that ST^{NR} infection increased mucus production in broilers. However, on D 21, supplemental TA increased goblet cell density in broilers infected with ST^{NR} along with enhanced ADG in the current study. Potentially, on D 21, increased mucus production by supplemental TA might have facilitated nutrient transportation, which led to increased growth performance. Increased mRNA expression of nutrient transporters (e.g., SGLT1 and EAAT3) by supplemental TA might be associated with enhanced mucus production on D 21 in the current study.

Regarding the timeline to assess the chicken host immune response, D 18 was selected as birds infected with ST^{NR} were previously reported to have impaired immunity and growth

performance in broilers (Adhikari et al., 2020). In the current study, the percentage of peripheral blood $CD8^+$ cells and the CD4:CD8 ratio were linearly increased and decreased, respectively along with enhanced ADG with increased TA supplementation in D 18 birds. An elevated percentage of $CD8^+$ cells is correlated with host immune response to bacterial infection (Jarosz et al., 2016) through enhanced T cell signaling and cytotoxicity (Fresnay et al., 2016). Thus, in the presence of an ST^{NR} infection, dietary supplementation with TA appears to favorably enhance cytotoxic CD8 mobility inferring some level of host cell mediated response. The number of lymphocytes, which are responsible for adaptive immunity, was quadratically decreased, and the TA0.5 group had the lower number of lymphocytes compared to the TA0 group. A decreased number of lymphocytes indicates attenuated inflammation (Guyton and Hall, 1996). Consistent with the present study, Ramirez and Roa Jr (2003) reported that tannin extract showed gastroprotective effects via a reduction in lymphocyte numbers in rats.

In the current study, there was also a linear increase in the number of monocytes in the enriched leukocyte fraction. Monocytes belong to innate immunity and play an important role in rapid response to pathogenic infection by producing cytokines, exhibiting phagocytic activities, and presenting antigens for further immune response (Stabler et al., 1994). Therefore, increased numbers of monocytes may represent enhanced innate immunity against pathogenic infection (Shang et al., 2015a). These findings are supported by Ramah et al. (2020), who observed that dietary TA supplementation enhanced broiler immunity. Overall, TA supplementation was demonstrated to positively affect innate and adaptive immunity in broilers.

The effects of different doses of supplemental TA on broilers infected with ST^{NR} are summarized in Table 5.12. The TA can show either negative or positive effects (e.g., hormesis) on broiler growth and health depending on the concentrations. Our current study showed that 1 to 2 g/kg supplemental TA exhibited beneficial effects in reducing ST^{NR} colonization in the ceca and stimulating immune system, which potentially resulted in improved growth performance in broilers infected with ST^{NR}. Our previous study showed that 0.5 to 2.75 g/kg supplemental TA had potential to be an anti-coccidial agent in broilers infected with *Eimeria maxima* (Choi et al., 2022b). However, supplemental TA higher than 1 g/kg exhibited anti-nutritional effects on broilers under non-challenge conditions (Choi et al., 2022c). Therefore, 1 g/kg supplemental TA would be appropriate would be appropriate to be an alternative for AGP in broiler production.

Table 5.12. Summary of effects of supplemental tannic acid on broilers infected with *Salmonella* Typhimurium nalidixic acid resistant strain (ST^{NR}).

Treatment	Observations
TA0.25	Decreased growth performance D 7 Decreased ST^{NR} liver colonization D 14 Decreased serum endotoxin D 21 Increased ileal villus height D 21
TA0.5	Decreased growth performance D 7 Increased gut permeability D 14 Increased growth performance D 21 Decreased ST^{NR} salmonella liver colonization D 14 Decreased serum endotoxin D 21 Increased serum alkaline activities D 21
TA1	Decreased growth performance D 7 Increased growth performance D 21 Decreased ST^{NR} salmonella cecal colonization D 7 Decreased serum endotoxin D 21 Increased serum alkaline activities D 21 Stimulated immune system D 21
TA2	Decreased growth performance D 7 Increased growth performance D 21 Decreased ST^{NR} salmonella cecal colonization D 7 Increased goblet cell density D 21 Increased serum alkaline activities D 21 Increased gene expression of nutrient transporters D 21 Stimulated immune system D 21

5.6. CONCLUSION

ST^{NR} infection resulted in decreased growth performance, increased gut permeability, increased the serum endotoxin concentrations, negatively affected immune parameters, decreased ileal morphology, modulated mucus production, and decreased nutrient absorption in broilers. However, supplemental TA led to reduced ST^{NR} colonization, increased growth performance, decreased the serum endotoxin concentrations, improved immune responses that were diminished by ST^{NR}, enhanced ileal morphology, increased mucus production, and increased nutrient absorption in broilers infected with ST^{NR}. Therefore, supplemental TA (1 to 2 g/kg) increased growth performance and gut health via antimicrobial and immunostimulatory effects in broilers infected with ST^{NR}. Supplemental TA can therefore be a potential alternative to AGP in broiler production.

6.0. CHAPTER 6 MANUSCRIPT IV

EFFECTS OF TANNIC ACID SUPPLEMENTATION ON GROWTH PERFORMANCE, OOCYST SHEDDING, AND GUT HEALTH OF IN BROILERS INFECTED WITH EIMERIA MAXIMA[1]

[1]**Choi, J.**, Y. H. Tompkins, P.-Y. Teng, R. M. Gogal Jr, and W. K. Kim. 2022. Effects of Tannic Acid Supplementation on Growth Performance, Oocyst Shedding, and Gut Health of in Broilers Infected with Eimeria Maxima. Animals 12:1378. Reprinted here with permission of the publisher.

6.1. ABSTRACT

The purpose of this study was to evaluate effects of tannic acid (TA) on growth performance, fecal moisture content, oocyst shedding, gut permeability, lesion score, intestinal morphology, apparent ileal digestibility, and the antioxidant and immune system of broilers infected with *Eimeria maxima*. A total of 420 one-day-old broilers were distributed to five treatments with seven replicates of 12 birds. The five treatments were the (1) sham-challenged control (SCC; birds fed a control diet and administrated with PBS); (2) challenged control (CC; birds fed a control diet and inoculated with *E. maxima*); (3) tannic acid 0.5 (TA0.5; CC + 500 mg/kg TA); (4) tannic acid 2.75 (TA2.75; CC + 2750 mg/kg TA); and (5) tannic acid 5 (TA5; CC + 5000 mg/kg TA). The TA2.75 group had significantly lower gut permeability compared to the CC group at 5 days post-infection (dpi). TA supplementation linearly reduced oocyst shedding of *E. maxima* at 7 to 9 dpi ($P < 0.05$). At 13 dpi, the TA2.75 group had significantly greater apparent ileal digestibility (AID) of dry matter (DM) and organic matter (OM) compared to the CC group. At 13 dpi, TA supplementation linearly increased jejunal villus height (VH) ($P < 0.05$). Thus, this study showed that TA supplementation at concentrations of 500 to 2750 mg/kg has the potential to be an anti-coccidial agent against *E. maxima* in broilers.

Keywords: Eimeria maxima; tannins; tannic acid; broilers; gut health; oocyst shedding

6.2. INTRODUCTION

Coccidiosis, induced by the apicomplexan protozoan parasites of the genus Eimeria, accounts for more than USD 3 billion in economic loss in the poultry industry annually (Abdullahi et al., 2020). The nine identified Eimeria spp. in chickens include *E. acervulina*, *E. brunetti*, *E. maxima*, *E. necatrix*, *E. praecox*, *E. mitis*, *E. tenella*, *E. mivati*, and *E. hagani* to

date (Chapman, 2014; Clark et al., 2016). *Eimeria* spp. infect and multiply within the mucosal epithelial layers in the different parts of the gastrointestinal tract of chickens (Li et al., 2019a). After several sets of asexual and sexual replications, oocysts are excreted with feces, can be sporulated in the appropriate environment (temperature, humidity, and access to oxygen), and infect chickens repeatedly when they are ingested by chickens (Waldenstedt et al., 2001). Infection of *E. maxima*, which propagates in the jejunum, can cause severe reduction in nutrient digestion and absorption along with inflammation caused by immune system in broilers (Teng et al., 2020b; Yin et al., 2014). Currently, prophylactic coccidiostats and anti-coccidial drugs are provided to control coccidiosis in broilers (Peek and Landman, 2011). However, due to the concern of spread of resistant bacteria and Eimeria strains, numerous studies have been conducted to find alternatives for anticoccidial drugs including (Teng et al., 2021b), prebiotics (Levine et al., 2018), plant extracts (Yadav et al., 2022b), organic acids (Abbas et al., 2011), and nitro compounds (Teng et al., 2020a) in poultry production.

Tannins, polyphenol compounds that can precipitate proteins, are categorized into hydrolysable and condensed tannins. Diverse tannin sources such as chestnut (Castanea sativa; hydrolysable tannin) and quebracho (Schinopsis lorentzii; condensed tannin) are known to control Eimeria infections (Cejas et al., 2011; Hooge et al., 2012; McCann et al., 2006). Hydrolysable and condensed tannins have different bioavailability because condensed tannins cannot be hydrolyzed into small molecules in chickens (Lu et al., 2020b). Although high doses (>5 g/kg) of tannins have cytotoxicity and are considered as anti-nutritional factors in chickens, tannins at appropriate concentrations are also known to show beneficial effects by exhibiting strong antimicrobial, antioxidant, and anti-inflammatory effects in chickens (Lu et al., 2020b).

Tannins can limit the growth of microorganisms by directly inhibiting activities of microbial enzymes and by indirectly forming complex with metal ions (Chung et al., 1998; Scalbert, 1991). Moreover, immunomodulatory and antioxidant properties of tannins have potentials to reduce the parasitic infection and attenuate negative impacts of parasitic infections, respectively, in chickens (Hamiza et al., 2012; Kaur et al., 2008). Tannic acid (TA) is considered as the typical and standard of hydrolysable tannins. Tonda et al. (2018) reported that supplementation of 500 mg/kg gallnut TA extract reduced total oocysts in excreta and decreased intestinal lesion scores in broilers infected with *E. acervulina*, *E. maxima*, and *E. tenella*. In contrast, Mansoori and Modirsanei (2012) showed that supplemental TA (10 g/kg) increased the total number of oocysts in excreta in broilers infected with *Eimeria* spp., which indicates that high concentrations of tannins can impair the immune system against coccidiosis and create better gut environment for Eimeria propagation. Whether TA supplementation at appropriate concentrations can show antiparasitic effects against *E. maxima* is still unknown. Hence, the purpose of the study was to evaluate effects of different concentrations of supplemental TA on growth performance, oocyst shedding, gut permeability, intestinal morphology, number of goblet cells, immune system, and antioxidant capacity in broilers infected with *E. maxima*.

6.3. MATERIALS AND METHODS

6.3.1. Preparation of *E. maxima* inoculum

The inoculum of *E. maxima* was freshly prepared before the inoculation according to the work of Teng et al. (2021a). Fresh fecal samples were collected from birds challenged with

E. maxima in the previous experiments and stored at 4 ◦C for further steps. To extract *E. maxima* from feces, water was added to the feces and blended thoroughly. Later, feces were removed from blended samples using cheesecloth, and solution was collected. The obtained solution was sedimented overnight and centrifuged at 1,000 × *g* for 10 min at room temperature to remove the supernatant. Afterwards, the saturated salt buffer was added up to 60% of the bottle. The solution was vortexed to suspend all oocysts and then sedimented at room temperature for 30 min. The final samples were centrifuged at 500 × *g* for 10 min, and oocysts were collected from the top phase of the supernatant into the new tubes. Water was added to the dilute salt solution and centrifuged at 1,000 × *g* for 10 min, and the supernatant was discarded. Finally, 2.5% potassium dichromate was added to suspended oocysts, and air was pumped for several days to sporulate the oocysts and the solution was subsequently stored at 4◦C for further steps. One day before the inoculation, 10^4 per 1 mL of PBS of *E. maxima* was prepared. To wash 2.5% potassium dichromate, the *E. maxima* solution was centrifuged at 1,000 × *g* for 10 min, and the supernatant was discarded. The PBS was added and centrifuged at 1,000 × *g* for 10 min, and the supernatant was discarded. This step was repeated twice to remove potassium dichromate. By using a hemocytometer (Hausser Scientific Company, Horsham, PA), the number of sporulated *E. maxima* were counted, and the appropriate amount of PBS was added to make 10^4 *E. maxima* per mL.

6.3.2. Experimental design and growth performance

This current study was approved by the Institutional Animal Care and Use Committee at the University of Georgia, Athens, GA. A total of 420 one-day-old Cobb 500 male broilers were randomly allocated to 5 treatments with 7 replicates of 12 birds in each battery cage. As

shown in Table 6.1., diets were formulated to meet or exceed energy and nutrient requirements according to the Cobb Broiler Management Guide (Cobb 2018). The TA was purchased from Sigma-Aldrich (St. Louis, MO) and was added into the filler part with sand to obtain the desired concentrations of TA in the feed. The five experimental treatments were: (1) sham-challenged control (SCC; birds fed a control diet and administrated with PBS); (2) challenged control (CC; birds fed a control diet and inoculated with *E. maxima*); (3) tannic acid 0.5 (TA0.5; CC + 500 mg/kg TA); (4) tannic acid 2.75 (TA2.75; CC + 2750 mg/kg TA); and (5) tannic acid 5 (TA5; CC + 5000 mg/kg TA). Experimental diets were provided during the whole experimental period. Birds had free access to water and feed during the entire experiment period (D 0 to 28), and temperature and light were maintained in accordance with the Cobb Broiler Management Guide (Cobb 2018). Birds and their living conditions were monitored twice daily. For implementing the *E. maxima* challenge model, 104/mL of sporulated *E. maxima* was inoculated via oral gavage to an individual bird on D 15. Body weight (BW) and feed disappearance were recorded to calculate average daily gain (ADG), average daily feed intake (ADFI), and feed conversion ratio (FCR) on D 15, 6 days post-infection (dpi) for the acute phase, and 13 dpi for the recovery phase.

Table 6.1. Ingredients and nutrient compositions of basal diets (As-fed basis)

Items	D 0 to 15	D 15 to 28
Ingredients (kg/ton)		
Corn	651.95	700.8
Soybean meal (480 g crude protein /kg)	294.94	241.76
Deflourinated phosphate	15.78	15.84
Filler[1] (sand and tannic acid)	10	13.99
Soybean oil	7.93	10
Limestone	7.16	6.11
DL-Methionine 99%	3.17	2.85
L-Lysine HCl 78%	3.01	2.8
Vitamin Premix[2]	2.5	2.5
Sodium chloride	1.55	1.79
L-threonine	1.2	0.8
Mineral Premix[3]	0.8	0.77
Total	1,000	1,000
Calculated energy and nutrient value, %		
Metabolizable energy, Mcal/kg	3,000	3,100
Crude protein, %	20.6	18.37
SID[4] Methionine, %	0.61	0.552
SID[4] Total sulfur amino acids, %	0.88	0.8
SID[4] Lysine, %	1.17	1.02
SID[4] Threonine, %	0.78	0.66
Total calcium, %	0.87	0.76
Available phosphorus, %	0.44	0.38

[1]Sand and tannic acid were added to obtain desired tannic acid concentration in the feed as follows (Control: sand 10 g/kg + tannic acid 0 g/kg, TA0.5: sand 9.5 g/kg + tannic acid 0.5 g/kg, TA2.75: sand 7.25 g/kg + tannic acid 2.75 g/kg, and TA5: sand 5 g/kg + tannic acid 5 g/kg).

[2]Vitamin mix provided the following in mg/100 g diet: thiamine-HCl, 1.5; riboflavin 1.5; nicotinic acid amide 15; folic acid 7.5; pyridoxine-HCl, 1.2; d-biotin 3; vitamin B-12 (source concentration, 0.1%) 2; d-calcium pantothenate 4; menadione sodium bisulfite, 1.98; α-

tocopherol acetate (source 500,000 IU/g), 22.8; cholecalciferol (source 5,000,000 IU/g) 0.09; retinyl palmitate (source 500,000 IU/g), 2.8; ethoxyquin, 13.34; I-inositol, 2.5; dextrose, 762.2.

[3]Mineral mix provided the following in g/100 g diet: $Ca(H_2PO_4)_2 \cdot H_2O$, 3.62; $CaCO_3$, 1.48; KH_2PO_4, 1.00; Na_2SeO_4, 0.0002; $MnSO_4 \cdot H_2O$, 0.035; $FeSO_4 \cdot 7H_2O$, 0.05; $MgSO_4 \cdot 7H_2O$, 0.62; KIO_3, 0.001; NaCl, 0.60; $CuSO_4 \cdot 5H_2O$, 0.008; $ZnCO_3$, 0.015; $CoCl_2 \cdot 6H_2O$, 0.00032; $NaMoO_4 \cdot 2H_2O$, 0.0011; KCl, 0.10; dextrose, 0.40.

[4]SID: standard ileal digestible amino acid.

6.3.3. Oocysts shedding and fecal consistency

Fresh fecal samples were collected in sample bags on 3 to 5, 5 to 7, 7 to 9, 9 to 11, and 11 to 13 dpi and stored at 4◦C for further steps. Oocyst shedding was measured according to the work of Teng et al. (2020c). Approximately 20 g of feces was weighed and vortexed thoroughly with 20 mL distilled water. One milliliter of samples was mixed with 9 mL of saturated salt solution and vortexed. Finally, the number of *E. maxima* oocysts was counted using a McMaster chamber (Vetlab Supply, Palmetto Bay, FL).

Within 3 to 4 days after sample collection, moisture contents of the ileal and fecal samples were determined as an indicator of fecal consistency according to the work of Koo et al. (2020). Approximately 10 to 15 g of the fecal sample was weighed into the plate and oven-dried at 120◦C for 12 h. Afterwards, the dried samples were weighed. The moisture contents (%) of fecal samples were calculated as follows:

$$\frac{Weight\ of\ the\ fecal\ sample\ before\ drying - Weight\ of\ the\ fecal\ sample\ after\ drying}{Weight\ of\ the\ fecal\ sample\ before\ drying} \times 100\ (\%)$$

6.3.4. *In vivo* gut permeability

At 5 dpi, gut permeability was measured using fluorescein isothiocyanate–dextran (molecular weight: 4 kda; FITC-D4; Sigma-Aldrich Co.) according to the work of Yadav et al. (2022b). The 2.2 mg/mL of FITC-D4 was prepared in PBS. One milliliter of the FITC-D4 solution was oral-gavaged to one bird per pen, and after sacrificing birds, blood was collected with heparin-free vacutainers (Grainer Bio-One, Kremsmuenster, Austria) from heart 2 h after FITC-F4 oral gavaging. Collected blood samples were stored in a dark container covered with

aluminum foil and kept at room temperature for 1 h to allow clotting. Afterwards, the blood samples were centrifuged at 1,000 × *g* for 10 min to recover serum. The serum (200 µL) was transferred to a dark 96-well plate, and the fluorescence was measured at an excitation wavelength of 485 nm and emission at 535 nm using a Spectra Max 5 microplate reader (Molecular Devices, Sunnyvale, CA). The concentrations of FITC-D4 were calculated using a prepared standard curve.

6.3.5. Tissue and digesta sample collection

At 6 dpi, four birds were euthanized by cervical dislocation, and the jejunal lesion from the mid-jejunum to ileum was scored according to the four-score scale described by Johnson and Reid (1970). At 6 and 13 dpi, a 2 cm segment of the mid-jejunum was collected and fixed in a 10% formaldehyde solution. A 10 cm segment of the mid-jejunum was removed and washed with PBS, immediately snap-frozen in liquid nitrogen, and stored at −80 ◦C for further analyses. Ileal digesta samples were obtained from the upper 10 cm of the ileo-cecal-colic junction to the 10 cm lower Meckel's diverticulum.

6.3.6. Total glutathione and oxidized glutathione assays, and total antioxidant capacity assay

Total glutathione (GSH) and oxidized glutathione (GSSG) of the mid-jejunal tissues and the liver were measured in duplicate using Caymans GSH assay kits (Cayman Chemical, Ann Arbor, MI). Briefly, 100 mg of mid-jejunal samples was weighted out in a 1.5 mL Eppendorf tube with liquid nitrogen, homogenized in 1 mL of phosphate solution (pH 6.8) containing 1 mM EDTA using a beads beater (Biospec Products, Bartlesville, OK) for 20 s, and then centrifuged at 10,000 × *g* for 15 min at 4 ◦C. Aliquots of the supernatants were taken for the

analyses of protein contents using Pierce™ BCA Protein Assay Kits (Thermo Fisher Scientific, Cleveland, OH) after 1:9 dilution. Afterward, metaphosphoric acid (10% w/v; sigma) was added to the obtained supernatant to precipitate protein, and then the mixture was centrifuged at 3,000 × *g* for 5 min at room temperature after vortexing. After deproteinization, total GSH and GSSG concentrations in the resulting supernatant were measured according to the manufacturer's protocol with following dilutions with sample buffer (jejunal GSH: 1 (sample):9 (buffer); jejunal GSSG 1:1; liver GSH 1:19; and liver GSSG 1:1). Reduced GSH was calculated by the equation: Reduced GSH = Total GSH − 2 × GSSG.

Total antioxidant capacity (TAC) of the mid-jejunal tissues was determined using colorimetric Microplate Assay Kits for Total Antioxidant Capacity (TA02, Oxford Biomedical Research, Oxford, MI) according to the work of Choi et al. (2020c) with some modifications. Briefly, 100 mg of mid-jejunal samples was weighted out in a 1.5 mL Eppendorf tube with liquid nitrogen, homogenized in 1 mL of PBS (pH 7.0) by a bead beater for 20 s, and then centrifuged at 3,000 × *g* for 15 min at 4 ◦C. Aliquots of the supernatants were taken for the analyses of their protein contents using Pierce BCA Protein Assay Kits (Thermo Fisher Scientific) with 1:9 dilution. The TAC analysis was conducted according to the manufacturer's protocol. The values were expressed as uric acid equivalent mM/mg protein.

6.3.7. Apparent ileal digestibility of nutrients

The concentrations of titanium dioxide in oven-dried samples (0.3 g for the ileal digesta samples and 0.5 g for the feed samples) were analyzed according to the work of Short et al. (1996). The CP content was determined by nitrogen combustion analysis (Method 990.03,

AOAC, 2006). Apparent ileal digestibility (AID) of DM, OM, CP, and ash were determined according to the work of Lin and Olukosi (2021).

6.3.8. Intestinal morphology and goblet cell counting analyses

The alcian blue/the period acid-Schiff (AB/PAS) staining was conducted to measure villus height (VH), crypt depth (CD), and the VH/CD ratio, as well as to count the number of goblet cells in VH and CD. Intestinal samples were fixed in 10% neutral-buffered formalin for 72 h and then were stored in the 70% alcohol for further analyses. Afterwards, the jejunal samples were cut. The sections were stained with alcian blue for 15 min and washed with distilled water. The samples were treated with periodic acid for 5 min and washed with distilled water. Subsequently, the samples were stained with Schiff's reagent for 10 min and washed with distilled water. Finally, the samples were counterstained in hematoxylin for 1 min and washed and dehydrated. The stained sections were pictured with a microscope (Leica DC500 camera, Leica Microsystems Inc., Buffalo Groove, IL). Images (4×) were analyzed using ImageJ (National Institutes of Health, Bethesda, MD).

6.3.9. RNA extraction and real-time reverse transcription (RT)-PCR analysis

Approximately 100 mg of the whole jejunum samples were homogenized in QIAzol lysis reagents (Qiagen, Valencia, CA), and RNAs were extracted according to the manufacturer's procedure. A NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific) was used to determine RNA quantity and purity. One microgram of RNA was used to produce the single-strand cDNA using high-capacity cDNA synthesis kits (Applied Biosystems, Foster City, CA). Primers used in the study are shown in Table 6.2. Real-time PCR was performed using SYBR

Green Master Mix (Invitrogen, Carlsbad, CA) with a Step One thermocycler (Applied Biosystem). The final PCR volume (10 µL) contained 5 µL of SYBR Green Master Mix, 1.5 µL of cDNA, 0.5 µL of forward and reverse primers (10 µM), and 2.5 µL of water. The thermal cycle condition for all reactions was as follows: 95 ◦C denature for 10 min, 40 cycles at 95 ◦C for 15 s and 60 ◦C for 1 min, 95 ◦C for 15 s, 60 ◦C for 1 min and 95 ◦C for 15 s. Several PCR products from each were electrophoresed on a 3% agarose gel in Tris-acetate-EDTA buffer and visualized by adding SYBR green (Invitrogen), and a melting curve of each gene was checked to confirm the specificity of each PCR product. The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and beta actin were used as the housekeeping genes (reference genes). The target mRNA abundance was normalized with geometric means of housekeeping genes (Vandesompele et al., 2002). Relative mRNA abundance was determined by using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The negative control, containing no cDNA, was included in each run, and each sample was run in duplicate.

Table 6.2. Primers used in the study

Genes	Sequence, 5′ to 3′	Amplicon
GAPDH	F: GCT AAG GCT GTG GGG AAA GT R: TCA GCA GCA GCC TTC ACT AC	161
Beta actin	F: CAA CAC AGT GCT GTC TGG TGG TA R: ATC GTA CTC CTG CTT GCT GAT CC	205
NFκB	F: GAA GGA ATC GTA CCG GGA ACA R: CTC AGA GGG CCT TGT GAC AGT AA	131
IL1β	F: TGC CTG CAG AAG AAG CCT CG R: GAC GGG CTC AAA AAC CTC CT	204
IL2	F: TTG GCT GTA TTT CGG TAG CA R: GTG CAC TCC TGG GTC TCA GT	169
IL6	F: ATA AAT CCC GAT GAA GTG G R: CTC ACG GTC TTC TCC ATA AA	146
JAM2	F: AGC CTC AAA TGG GAT TGG ATT R: CAT CAA CTT GCA TTC GCT TCA	59
ZO2	F: ATC CAA GAA GGC ACC TCA GC R: CAT CCT CCC GAA CAA TGC	100
CLDN4	F: GAA GCG CTG AAA CGA TAC CA R: TGC TTC TGT GCC TCA GTT TCC	134
SGLT1	F: GCC ATG GCC AGG GCT TA R: CAA TAA CCT GAT CTG TGC ACC AGT A	71
PEPT1	F: CCC CTG AGG AGG ATC CTT R: CAA AAG AGC AGC AAC GA	66
B0AT1	F: GGG TTT TGT GTT GGC TTA GGA A R: TCC ATG GCT CTG GCA GAG AT	60
EAAT3	F: TGC TGC TTT GGA TTC CAG TGT R: AGC AAT GAC TGT AGT GCA GAA GTA ATA TAT G	79
GLUT2	F: TCA TTG TAG CTG AGC TGT T R: CGA AGA CAA CGA ACA CAT AC	147
MUC2	F: ATG CGA TGT TAA CAC AGG ACT C R: GTG GAG CAC AGC AGA CTT TG	110

[1]GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; NFκB, nuclear factor kappa-light-chain-enhancer of activated B cells; IL, interleukin; JAM2, Junctional adhesion molecule 2; ZO2, zonula occludens 2; CLDN4, claudin 4; SGLT1, sodium glucose transporter 1; PepT1, peptide transporter 1; B0AT1, sodium-dependent neutral amino acid transporter 1; EAAT3, excitatory amino acid transporter 3; GLUT2, glucose transporter 2; MUC2, mucin 2.

6.3.10. Statistical analyses

Statistical analyses were conducted utilizing SAS (version 9.4; SAS Inst. Inc., Cary, NC). Data normality was checked using proc univariate except for lesion score data. The SCC and CC groups were compared using Student's t-test. *E. maxima*-infected groups were compared using one-way ANOVA in a completely randomized design followed by Tukey's comparison test. Orthogonal polynomial contrasts analysis was conducted to see linear pattern (L) and quadratic pattern (Q) among *Eimeria maxima* challenged groups. The Kruskal–Wallis test followed by the Dwass–Steel–Critchlow–Fligner post hoc test was performed to analyze lesion score data. Significance level was set at $P < 0.05$, and tendencies were also presented at $0.05 < P \leq 0.10$ (Choi et al., 2021).

6.4. RESULTS

6.4.1. Growth performance

As shown in Table 6.3., the TA5 birds had the reduced BW and ADG compared to the CC and TA0.5 groups ($P < 0.05$), and BW, ADG, and ADFI were linearly reduced by TA supplementation in the pre-challenge period ($P < 0.05$). TA supplementation tended to linearly increase FCR in broilers in the pre-challenge period ($P = 0.077$). In the acute phase (0 to 6 dpi), *E. maxima* infection significantly decreased BW, ADG, and ADFI in broilers. The TA0.5 group had the significantly higher BW compared to the TA5 group, and BW was linearly reduced as TA supplementation concentration increased ($P < 0.05$). In the recovery phase (6 to 13 dpi), the CC group had statistically similar BW compared to the SCC group ($P > 0.1$) but had significantly higher ADFI compared to the SCC broilers. The TA5 group had the reduced

BW at 13 dpi compared to the CC and TA0.5 groups ($p < 0.05$), and TA supplementation linearly reduced BW and ADG and increased FCR in broilers infected with *E. maxima* ($p < 0.01$).

Table 6.3. Effects of tannic acid supplementation on growth performance parameters including body weight (BW, g), average daily gain (ADG, g/d), average daily feed intake (ADFI, g/d), and feed conversion ratio (FCR, g/g) of broilers infected with *Eimeria maxima* during the pre-challenge period: 0 to 15 d, acute phase: 0 to 6 days post infection (dpi), and recovery phase: 6 to 13 dpi[1].

		Eimeria maxima-challenged[3]					Polynomial contrast	
Items	SCC[2]	CC	TA0.5	TA2.75	TA5	SEM	*P* value	Linear	Quadratic
Pre-challenge									
BW	462.1	440.8^{a}	452.8^{a}	432.8^{ab}	404.7^{b}	19.9	<0.001	<0.001	0.192
ADG	27.68	26.26^{a}	27.06^{a}	25.7^{ab}	23.8^{b}	1.4	0.001	<0.001	0.193
ADFI	38.96	38.37	38.46	37.11	35.85	2.02	0.076	0.011	0.877
FCR	1.41	1.46	1.42	1.44	1.50	0.11	0.123	0.077	0.161
Acute phase									
BW	812.2***	696.5^{ab}	721.4^{a}	691.9^{ab}	638.5^{b}	41.7	0.043	0.011	0.2
ADG	58.3***	42.6	44.7	43.2	39	5.5	0.618	0.328	0.405
ADFI	115.4***	86.8	87.5	92.3	87.9	7.51	0.518	0.594	0.177
FCR	1.99	2.08	1.96	2.18	2.33	0.48	0.805	0.356	0.904
Recovery phase									
BW	1273	1211^{a}	1227^{a}	1141^{ab}	1062^{b}	105.3	0.005	<0.001	0.413
ADG	69.8	75.6	74.2	64.3	57.6	23.5	0.042	0.005	0.817
ADFI	118**	130.2	128.1	124	121	11.7	0.473	0.125	0.828
FCR	1.75	1.75	1.76	1.94	2.2	0.32	0.045	0.005	0.724

[1]SCC (sham-challenged control): broilers fed a control diet and administrated with phosphate buffered saline via oral gavage; CC (Challenged control): broilers fed a control diet and administrated with 10^4 of *Eimeria maxima* via oral gavage; TA0.5 (Tannic acid 500 mg/kg): CC + 500 mg/kg of tannic acid; TA2.75 (Tannic acid 2,750 mg/kg): CC + 2,750 mg/kg of tannic acid; and TA5 (Tannic acid 5,000 mg/kg): CC + 5,000 mg/kg of tannic acid.

[2]SCC v. CC (unpaired t test): * $0.05 < P < 0.10$, ** $P < 0.05$, *** $P < 0.01$.

[3]*Eimeria maxima* challenged groups (CC, TA0.5, TA2.75 and TA5) were compared by PROC MIXED followed by the Tukey's multiple comparison test. Different letters in the same row means significant differences ($P < 0.05$) among treatments.

6.4.2. Fecal moisture content and oocyst shedding

Inoculation of *E. maxima* did not affect the ileal and fecal moisture contents at all time points, as shown in Table 6.4. ($P > 0.1$). At 13 dpi, TA supplementation linearly reduced ileal moisture content ($P < 0.05$). At 5 to 7 dpi, the TA2.75 group had a decreased fecal moisture content compared to the CC group, and TA supplementation linearly and quadratically reduced fecal moisture content ($P < 0.05$). At 9 to 11 dpi and 11 to 13 dpi, the TA5 group had decreased fecal moisture content compared to the TA0.5 group, and TA supplementation linearly reduced fecal moisture content ($P < 0.01$).

As shown in Table 6.5., in the SCC group, *E. maxima* oocysts were not detected in feces at all time points. At 5 to 7 dpi, TA supplementation tended to linearly reduce oocyst shedding in broilers ($P = 0.091$); much less *E. maxima* in the jejunal section of the TA0.5 group was observed compared to the CC group (Figure 6.1.). At 7 to 9 dpi, TA0.5, TA2.75, and TA5 groups had significantly lower oocyst shedding compared to the CC group. However, differences in oocyst shedding were not observed at 9 to 11 dpi among the treatments ($P > 0.1$).

Table 6.4. Effects of tannic acid supplementation on ileal and fecal moisture content (%) of broilers infected *Eimeria maxima* on 6- and 13- days post infection (dpi) for ileal samples and 3 to 5 dpi, 5 to 7 dpi, 7 to 9 dpi, 9 to 11 dpi and 11 to 13 dpi for fecal samples[1]

			Eimeria maxima-challenged[3]					Polynomial contrast	
Items	SCC[2]	CC	TA0.5	TA2.75	TA5	SEM	*P* value	Linear	Quadratic
Ileal content									
6 dpi	81.64	82.46	84.24	82.02	82.69	2.03	0.224	0.439	0.628
13 dpi	81.46	81.99	82.00	81.23	80.45	1.2	0.073	0.01	0.856
Fecal content									
3 to 5 dpi	75.47	75.30	78.23	75.68	76.09	4.44	0.619	0.769	0.966
5 to 7 dpi	78.84	76.77^{a}	74.84^{a}	68.69^{b}	72.41^{ab}	3.92	0.005	0.016	0.005
7 to 9 dpi	78.85	78.00	79.65	76.00	77.28	2.54	0.09	0.131	0.225
9 to 11 dpi	80.04	80.06^{ab}	82.11^{a}	79.74^{ab}	77.26^{b}	2.54	0.012	0.005	0.265
11 to 13 dpi	77.77	79.32^{a}	79.79^{a}	77.24^{ab}	74.80^{b}	2.70	0.008	<0.001	0.722

[1]SCC (sham-challenged control): broilers fed a control diet and administrated with phosphate buffered saline via oral gavage; CC (Challenged control): broilers fed a control diet and administrated with 10^4 of *Eimeria maxima* via oral gavage; TA0.5 (Tannic acid 500 mg/kg): CC + 500 mg/kg of tannic acid; TA2.75 (Tannic acid 2,750 mg/kg): CC + 2,750 mg/kg of tannic acid; and TA5 (Tannic acid 5,000 mg/kg): CC + 5,000 mg/kg of tannic acid.

[2]SCC v. CC (unpaired *t* test): * $0.05 < P < 0.10$, ** $P < 0.05$, *** $P < 0.01$.

[3]*Eimeria maxima* challenged groups (CC, TA0.5, TA2.75 and TA5) were compared by PROC MIXED followed by the Tukey's multiple comparison test. Different letters in the same row means significant differences ($P < 0.05$) among treatments.

Table 6.5. Effects of tannic acid supplementation on oocyst shedding (number of oocysts per gram in feces) in broilers infected *Eimeria maxima* on 5 to 7 days post infection (dpi), 7 to 9 dpi, and 9 to 11 dpi.

		Eimeria maxima-challenged[3]						Polynomial contrast	
Items	SCC[2]	CC	TA0.5	TA2.75	TA5	SEM	*P* value	Linear	Quadratic
5 to 7 dpi		2205.6	2198.6	371.3	426.1	2446.8	0.308	0.091	0.471
7 to 9 dpi	N/D[4]	6203.8[a]	686.3[b]	680.8[b]	452.1[b]	3719.2	0.019	0.041	0.148
9 to 11 dpi		827.2	1143.6	272.9	963.8	1140.7	0.528	0.784	0.266

[1]SCC (sham-challenged control): broilers fed a control diet and administrated with phosphate buffered saline via oral gavage; CC (Challenged control): broilers fed a control diet and administrated with 10^4 of *Eimeria maxima* via oral gavage; TA0.5 (Tannic acid 500 mg/kg): CC + 500 mg/kg of tannic acid; TA2.75 (Tannic acid 2,750 mg/kg): CC + 2,750 mg/kg of tannic acid; and TA5 (Tannic acid 5,000 mg/kg): CC + 5,000 mg/kg of tannic acid.

[2]SCC v. CC (unpaired *t* test): * $0.05 < P < 0.10$, ** $P < 0.05$, *** $P < 0.01$.

[3]*Eimeria maxima* challenged groups (CC, TA0.5, TA2.75 and TA5) were compared by PROC MIXED followed by the Tukey's multiple comparison test. Different letters in the same row means significant differences ($P < 0.05$) among treatments.

[4]N/D: not-detected.

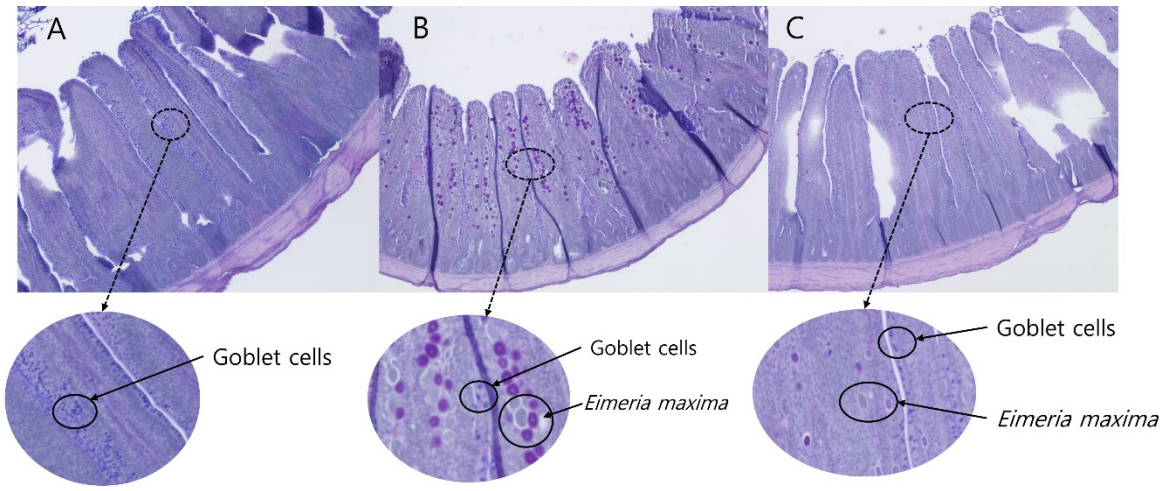

Figure 6.1. Alcian blue/the period acid-schiff (AB/PAS) stained jejunal morphology of A) SCC (sham-challenged control): chickens fed a control diet and challenged with phosphate buffered saline, B) CC (challenged control): chickens fed a control diet and challenged with 10^4 of *Eimeria maxima*; and C) TA0.5 (tannic acid 500 mg/kg): CC + 500 mg/kg tannic acid. Small purple dots are goblet cells and big white and purple dots are *E. maxima*. The SCC group had no *E. maxima* cross-contamination, and the TA0.5 group had less *E. maxima* in the mucus layer compared to the CC group.

6.4.3. Gut permeability and jejunal lesion

The inoculation of *E. maxima* increased the serum FITC-D4 concentration in broilers ($P < 0.05$), which indicates higher gut leakage (Table 6.6.). The TA2.75 group had significantly reduced serum FITC-D4 concentrations than the CC group, and TA supplementation quadratically reduced serum FITC-D4 concentrations ($P < 0.05$). As shown in Table 6.6., *E. maxima* inoculation induced mild jejunal lesion in broilers ($P < 0.05$). However, TA supplementation did not modulate jejunal lesion in broilers infected with *E. maxima* ($p > 0.1$).

Table 6.6. Effects of tannic acid supplementation on serum FITC D4 concentration and *Eimeria maxima* lesion in broiler chickens infected with *Eimeria maxima* on 5 days post-infection (dpi) and 6 dpi, respectively[1]

		Eimeria maxima-challenged[3]					Polynomial contrast	
Items	SCC[2]	CC	TA0.5	TA2.75	TA5	SEM	*P* value	Linear	Quadratic
Serum FITC-D4	0.15***	2.22[a]	1.76[ab]	1.06[b]	2.04[a]	0.10	0.003	0.463	<0.001
Jejunal lesion[4]	0***	0.75	0.96	0.78	0.82	0.02	0.579		

[1]SCC (sham-challenged control): broilers fed a control diet and administrated with phosphate buffered saline via oral gavage; CC (Challenged control): broilers fed a control diet and administrated with 10^4 of *Eimeria maxima* via oral gavage; TA0.5 (Tannic acid 500 mg/kg): CC + 500 mg/kg of tannic acid; TA2.75 (Tannic acid 2,750 mg/kg): CC + 2,750 mg/kg of tannic acid; TA5 (Tannic acid 5,000 mg/kg): CC + 5,000 mg/kg of tannic acid.

[2]SCC v. CC (unpaired *t* test): * $0.05 < P < 0.10$, ** $P < 0.05$, *** $P < 0.01$.

[3]*Eimeria maxima* challenged groups (CC, TA0.5, TA2.75 and TA5) were compared by PROC MIXED followed by the Tukey's multiple comparison test. Different letters in the same row means significant differences ($P < 0.05$) among treatments.

[4]The Kruskal-Wallis test followed by the Dwass, Steel, Critchlow-Fligner post-hoc test was used to analyze lesion score data.

6.4.4. Total antioxidant capacity, total glutathione, reduced glutathione, and oxidized glutathione

E. maxima infection did not affect TAC of the jejunum at 6 dpi ($P > 0.1$; Table 6.7). TA supplementation tended to linearly reduce TAC of the jejunum at 13 dpi ($p = 0.076$). At 6 dpi, TA supplementation linearly decreased total GSH ($P < 0.05$) and reduced GSH (tendency; $P = 0.075$) and increased GSSG (tendency; $P = 0.052$) in the jejunum of broilers infected with *E. maxima*. At 6 dpi, *E. maxima* infection tended to decrease reduced GSH ($p = 0.096$) and significantly decreased reduced GSH/GSSG in the liver ($P < 0.05$). At 13 dpi, the TA2.75 group had lower total GSH and reduced GSH in the jejunum compared to the TA0.5 group ($P < 0.05$), and the TA supplementation tended to linearly decrease total GSH ($p = 0.076$) and reduced GSH ($P = 0.10$) and linearly increase GSSG ($P = 0.10$) in the jejunum. At 13 dpi, *E. maxima* infection tended to increase GSSG ($P = 0.063$) and decreased reduced GSH/GSSG ($P = 0.064$) in the liver. The reduced GSH in the liver tended to be linearly decreased due to TA supplementation ($P = 0.098$).

Table 6.7. Effects of tannic acid supplementation on total antioxidant capacity (TAC) in the jejunum and glutathione (GSH), oxidized GSH (GSSG), reduced GSH, and the reduced GSH:GSSG ratio in the jejunum and liver on 6 days post infection (dpi) and 13 dpi in broiler chickens infected with *Eimeria maxima*[1]

			Eimeria maxima-challenged[3]					Polynomial contrast	
Items	SCC[2]	CC	TA0.5	TA2.75	TA5	SEM	*P* value	Linear	Quadratic
6 dpi jejunum									
TAC	73.67	76.98	73.62	75.93	73.12	7.27	0.72	0.538	0.798
Total GSH	42.57	31.37	30.53	24.12	20.39	10.31	0.171	0.029	0.808
GSSG	2.81	3.38	1.49	1.31	1.37	1.42	0.033	0.052	0.13
Reduced GSH[4]	36.93	24.61	27.55	21.50	17.66	9.69	0.286	0.075	0.844
Reduced GSH:GSSG	14.67	12.77	20.03	18.32	14.61	7.98	0.322	0.857	0.205
Liver									
Total GSH	52.58	43.5	33.38	31.07	33.6	13.60	0.346	0.296	0.256
GSSG	2.35	3.13	2.36	2.42	2.06	1.47	0.586	0.285	0.823
Reduced GSH	47.87*	37.24	28.65	26.23	29.47	11.94	0.362	0.353	0.216
Reduced GSH:GSSG	29.43**	13.16	13.23	14.16	17.54	9	0.777	0.33	0.74
13 dpi Jejunum									
TAC	112.1	101.6	101.8	91.38	87.04	17.99	0.337	0.076	0.805
Total GSH	53.45	50.57[ab]	61.24[a]	35.07[b]	46.54[ab]	13.81	0.015	0.071	0.063
GSSG	5.45	4.70	5.20	3.57	4.10	1.30	0.133	0.10	0.226
Reduced GSH	42.54	41.18[ab]	50.83[a]	27.9[b]	38.33[ab]	12.75	0.023	0.10	0.076
Reduced GSH:GSSG	7.87	11.95	9.75	8.89	9.84	6.31	0.828	0.614	0.508
Liver									
Total GSH	40	44.15	41.37	38.95	39.3	5.36	0.296	0.098	0.306

GSSG	4*	5.95	5.01	5.5	5.49	2.18	0.883	0.954	0.81
Reduced GSH	32	32.24	31.35	27.96	28.31	5.14	0.323	0.10	0.386
Reduced GSH:GSSG	8.56*	5.93	6.31	5.99	5.84	1.78	0.963	0.767	0.879

[1]SCC (sham-challenged control): broilers fed a control diet and administrated with phosphate buffered saline via oral gavage; CC (Challenged control): broilers fed a control diet and administrated with 10^4 of *Eimeria maxima* via oral gavage; TA0.5 (Tannic acid 500 mg/kg): CC + 500 mg/kg of tannic acid; TA2.75 (Tannic acid 2,750 mg/kg): CC + 2,750 mg/kg of tannic acid; and TA5 (Tannic acid 5,000 mg/kg): CC + 5,000 mg/kg of tannic acid.

[2]SCC v. CC (unpaired *t* test): * $0.05 < P < 0.10$, ** $P < 0.05$, *** $P < 0.01$.

[3]*Eimeria maxima* challenged groups (CC, TA0.5, TA2.75 and TA5) were compared by PROC MIXED followed by the Tukey's multiple comparison test. Different letters in the same row means significant differences ($P < 0.05$) among treatments.

[4]Reduced GSH = Total GSH – 2 × GSSG.

6.4.5. Apparent ileal digestibility of nutrients

As shown in Table 6.8., *E. maxima* infection significantly increased AID of ash, and the TA0.5 and TA5 groups had lower AID of ash compared to the CC group at 6 dpi. At 6 dpi, the TA5 group had lower AID of ash compared to the TA2.75 group. TA supplementation linearly decreased AID of ash at 6 dpi ($P < 0.05$). However, no differences were observed in AID of DM and OM among the treatments at 6 dpi ($P > 0.1$). At 13 dpi, the CC broilers had significantly greater AID of DM, OM, and ash compared to the SCC broilers ($P < 0.05$). The TA2.75 group had significantly higher AID of DM and OM compared to the CC group. However, the TA5 group had the lowest AID of DM and OM among the *E. maxima*-infected broilers. The TA0.5 and TA5 groups had significantly lower AID of ash compared to the CC and TA2.75 groups. No differences were observed in AID of CP at 13 dpi among the treatments ($P > 0.1$). TA supplementation linearly decreased AID of DM, OM, and ash in broilers infected with *E. maxima* ($P < 0.05$).

Table 6.8. Effects of tannic acid supplementation on apparent ileal digestibility of dry matter (DM), organic matter (OM), ash and crude protein (CP) on 6 days post infection (dpi) and 13 dpi in broilers infected with *Eimeria maxima*.

			Eimeria maxima-challenged[2]					Polynomial contrast	
Items	SCC	CC	TA0.5	TA2.75	TA5	SEM	*P* value	Linear	Quadratic
6 dpi									
DM	59.9	63.06	58.84	62.32	59.69	6.98	0.626	0.721	0.784
OM	65.45	65.45	61.95	65.04	63.93	6.67	0.765	0.941	0.937
Ash	12.5**	29.18^{a}	0.93^{bc}	12.33^{ab}	-22.66^{c}	16.64	<0.001	<0.001	0.196
CP	72	71.2	64.17	69.3	67.9	5.85	0.173	0.999	0.875
13 dpi									
DM	69**	73.85^{b}	74.88^{ab}	77.05^{a}	69.67^{c}	1.79	<0.001	<0.001	<0.001
OM	72.05**	75.73^{b}	77^{ab}	78.8^{a}	71.6^{c}	1.77	<0.001	<0.001	<0.001
Ash	17.86***	47.14^{a}	37.54^{b}	44.87^{a}	32.2^{b}	4.26	<0.001	<0.001	0.011
CP	68.59	73.3	68.32	69.14	68.03	5.73	0.303	0.231	0.585

[1]SCC (sham-challenged control): broilers fed a control diet and administrated with phosphate buffered saline via oral gavage; CC (Challenged control): broilers fed a control diet and administrated with 10^4 of *Eimeria maxima* via oral gavage; TA0.5 (Tannic acid 500 mg/kg): CC + 500 mg/kg of tannic acid; TA2.75 (Tannic acid 2,750 mg/kg): CC + 2,750 mg/kg of tannic acid; and TA5 (Tannic acid 5,000 mg/kg): CC + 5,000 mg/kg of tannic acid.

[2]SCC v. CC (unpaired *t* test): * $0.05 < P < 0.10$, ** $P < 0.05$, *** $P < 0.01$.

[3]*Eimeria maxima* challenged groups (CC, TA0.5, TA2.75 and TA5) were compared by PROC MIXED followed by the Tukey's multiple comparison test. Different letters in the same row means significant differences ($P < 0.05$) among treatments.

6.4.6. Jejunal morphology and goblet cell density

Infection of *E. maxima* significantly reduced jejunal VH and the VH/CD ratio when CC birds were compared to the SCC birds at 6 dpi (Table 6.9. and Figure 6.1.). At 13 dpi, jejunal VH and VH/CD ratio were decreased by E. maxima infection ($P < 0.05$), whereas jejunal CD and goblet cells per 100 µm VH and per 100 µm CD of the CC group were increased compared to the SCC group ($P < 0.05$). TA supplementation linearly increased jejunal VH in broilers infected with E. maxima ($P < 0.05$).

Table 6.9. Effects of tannic acid supplementation on villus height (VH, μm), crypt depth (CD, μm), VH:CD, goblet cells per 100 μm VH, goblet cells per 100 μm CD in the jejunum on 6 days post infection (dpi) and 13 dpi in broilers infected with *Eimeria maxima*

			Eimeria maxima-challenged[3]					Polynomial contrast	
Items	SCC[2]	CC	TA0.5	TA2.75	TA5	SEM	*P* value	Linear	Quadratic
6 dpi									
VH	1375***	775.6	812.26	764.79	739.04	191.88	0.912	0.556	0.901
Goblet cells per 100 μm VH	7.48	7.82	7.63	7.37	9.15	1.7	0.232	0.141	0.146
CD	248.5	279.22	357.8	354.94	328.46	55.56	0.05	0.385	0.056
Goblet cells per 100 μm CD	4.05	3.94	3.74	3.62	3.97	0.49	0.5	0.831	0.145
VH:CD	5.8***	2.87	2.49	2.22	2.39	0.82	0.508	0.317	0.303
13 dpi									
VH	1344**	973.9	1101.3	1210.8	1299.3	245.9	0.104	0.02	0.592
Goblet cells per 100 μm VH	5.12***	8.53	10.12	8.35	8.67	1.75	0.244	0.418	0.835
CD	248.62**	310.36	289.7	315.88	329.65	42.68	0.208	0.109	0.74
Goblet cells per 100 μm CD	2.72***	4.46	4.34	4.14	3.87	0.96	0.694	0.241	0.992
VH:CD	5.81***	3.22	4.09	3.95	4.07	0.85	0.205	0.212	0.456

[1]SCC (sham-challenged control): broilers fed a control diet and administrated with phosphate buffered saline via oral gavage; CC (Challenged control): broilers fed a control diet and administrated with 10^4 of *Eimeria maxima* via oral gavage; TA0.5 (Tannic acid 500 mg/kg): CC + 500 mg/kg of tannic acid; TA2.75 (Tannic acid 2,750 mg/kg): CC + 2,750 mg/kg of tannic acid; and TA5 (Tannic acid 5,000 mg/kg): CC + 5,000 mg/kg of tannic acid.

[2]SCC v. CC (unpaired *t* test): * $0.05 < P < 0.10$, ** $P < 0.05$, *** $P < 0.01$.

[3]*Eimeria maxima* challenged groups (CC, TA0.5, TA2.75 and TA5) were compared by PROC MIXED followed by the Tukey's multiple comparison test. Different letters in the same row means significant differences ($P < 0.05$) among treatments.

6.4.7. Relative mRNA expression

Due to *E. maxima* infection, relative mRNA expression of toll-like receptor 4 (TLR4) was significantly reduced, and relative mRNA expression of zonula occludens 2 (ZO2), claudins 4 (CLDN4), and mucin 2 (MUC2) of the CC group tended to be decreased ($p = 0.052$, $p = 0.074$, and $p = 0.056$, respectively; Figure 6.2.) at 6 dpi. TA supplementation tended to quadratically increase relative mRNA expression of interleukin 1β (IL1β; $p = 0.096$), nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB; $p = 0.071$), and junctional adhesion molecule 2 (JAM2; $p = 0.083$) at 6 dpi. During the recovery phase, the CC broilers had significantly lower relative mRNA expression of sodium-dependent neutral amino acid transporter (B0AT1) and excitatory amino acid transporter 3 (EAAT3) compared to the SCC broilers. Nevertheless, no differences were observed among the *E. maxima* infected groups.

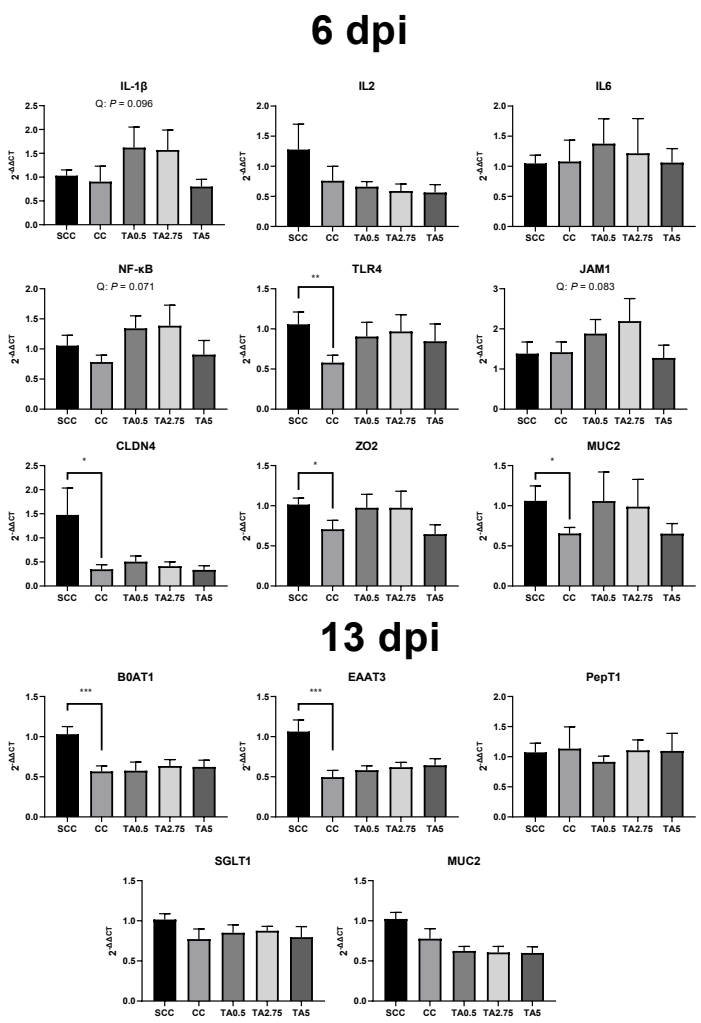

Figure 6.2. Relative mRNA gene expression in the jejunum on 6 days post infection (dpi) (genes related to immune system, gut barrier integrity and mucin) and 13 dpi (nutrient transporter and mucin) in the SCC [(sham-challenged control): broilers fed a control diet and administrated with phosphate buffered saline via oral gavage]; CC [(Challenged control): broilers fed a control diet and administrated with 10^4 of *Eimeria maxima* via oral gavage]; TA0.5 [(Tannic acid 500 mg/kg): CC + 500 mg/kg of tannic acid]; TA2.75 [(Tannic acid 2,750 mg/kg): CC + 2,750 mg/kg of tannic acid]; TA5 [(Tannic acid 5,000 mg/kg): CC + 5,000 mg/kg of tannic acid] groups. Each value represents means ± SEM. SCC was compared with

SC (unpaired t-test), and the comparison was presented as *0.05 < P < 0.10, **P < 0.05, ***P < 0.01. Bars with different letters are significantly different (P < 0.05) by PROC MIXED followed by the Tukey's multiple comparison test among *E. maxima* infected groups (CC, TA0.5, TA2.75, and TA5). GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; NFκB, nuclear factor kappa-light-chain-enhancer of activated B cells; IL, interleukin; JAM2, Junctional adhesion molecule 2; ZO2, Zonula occludens 2; CLDN4, Claudin 4; SGLT1, sodium glucose transporter 1; PepT1, peptide transporter 1; B0AT1, Sodium-dependent neutral amino acid transporter 1; EAAT3, excitatory amino acid transporter 3; GLUT2, glucose transporter 2; and MUC2, mucin 2.

6.5. DISCUSSION

The purpose of the current study was to investigate effects of TA on growth performance, intestinal morphology, goblet cell number, gut barrier integrity, antioxidant capacity, oocyst shedding, and immune system in broilers infected with *E. maxima*. *E. maxima* infection significantly decreased BW, ADG, and ADFI in the acute phase, which is consistent with our previous study (Teng et al., 2021a). During the recovery phase, the CC group had statistically similar BW to the SCC group by enhancing feed intake and nutrient digestibility in the current study, which implies that there was compensatory growth in the CC group. TA supplementation reduced BW and ADG during the pre-challenge period via decreasing the feed intake. The TA–saliva protein complexes are known to induce astringent and bitter taste, which may decrease feed intake of broilers (Teng et al., 2021a). Moreover, compromised gut health due to the high concentrations of TA potentially reduces feed intake of broilers (Van der Aar et al., 2017). During the acute phase, TA supplementation linearly decreased BW of broilers without affecting feed intake, intestinal morphology, and nutrient digestibility of broilers, which suggests that reduced growth performance by TA supplementation at young ages may continuously affect growth performance of broilers at later growing phases. However, the TA0.5 group had the numerically highest BW among the *E. maxima*-infected groups, which suggests that supplementation of 500 mg/kg TA may have potential to improve growth performance of broilers infected with *E. maxima*. In contrast, TA supplementation linearly reduced BW, ADG, and FCR of broilers infected with *E. maxima* in the recovery phase. In the current study, TA supplementation did not reduce ADG, ADFI, and FCR in the acute phase potentially because TA exhibited defensive effects against *E. maxima* infection in broilers.

TA supplementation decreased oocyst shedding of *E. maxima* in broilers at 5 to 9 dpi. Consistent with a previous study (Cha et al., 2018), around 7 dpi was the peak point for oocyst shedding of *E. maxima*. Moreover, pictures of jejunum morphology showed visually less *E. maxima* in the TA0.5 group compared to the CC group (Figure 6.1.). In agreement with this, Tonda et al. (2018) and Kaleem et al. (2014) reported that tannin extract from plants decreased oocyst shedding in broilers infected with *Eimeria* spp. Because there were no differences in the jejunal lesion score among the groups infected with *E. maxima*, thereby, TA could potentially not inhibit the invasion of *E. maxima* into the enterocytes but instead inhibit the sexual reproduction of *E. maxima* to produce oocysts. Sexual reproduction of *Eimeria* spp., following at least two cycles of asexual reproductions, is required to produce oocysts (Walker et al., 2015). Potentially, TA may directly inhibit enzymes activities and interact with proteins related to sexual reproduction or deprive nutrients (e.g., iron and proteins) from parasites for their reproduction by forming complex with proteins and metals. Reducing oocyst shedding is an important trait as an anti-coccidial agent because this possibly reduces continuous exposure of *E. maxima* in a chicken flock (Cha et al., 2018).

We hypothesized that *E. maxima* infection may increase ileal and fecal moisture content because *E. maxima* is known to induce diarrhea in broilers (Miska and Fetterer, 2018). However, the inoculation concentration used in the current study induced only mild infection and did not lead to watery digesta and feces. Because *E. maxima* infection did not induce diarrhea in the present study, decreased ileal and fecal moisture content might not have been due to anti-diarrhea effects of TA. Reduced moisture content in ileal digesta (9 dpi) and feces (9 to 13 dpi) could be closely associated with drastically reduced AID of ash in the current study. Tannins form a complex with minerals (zinc, iron, copper, etc.), which potentially

results in decreased mineral utilization and increased endogenous loss of minerals in chickens (Karamać, 2009). Secretion and absorption of electrolytes are closely related to water secretion and absorption in the intestine, and ileal and fecal moisture contents were reduced with the similar trends with the AID of ash in the current study. Reducing digesta and fecal moisture content via reducing AID of ash are not considered beneficial effects because loss of ash can result in reduced growth performance and bone development in broilers.

Gut permeability, measured by the FITC-D4 gavage method, was increased by *E. maxima* infection, consistent with our previous study (Yadav et al., 2022b). Increased gut permeability, which is mainly modulated by tight junction proteins (ZO2, CLDN4, JAM2, etc.) and mucus (main protein in the intestine, MUC2) (Liu et al., 2021), indicates that pathogenic bacteria and toxins are more likely to enter the bloodstream of broilers (Liu et al., 2021). Consistent with the FITC-D4 permeability assay result, *E. maxima* infection reduced mRNA expression of ZO2, CLDN4, and MUC2 in the present study. Teng et al. (2021a) reported that during the *E. maxima* asexual and sexual replication, tight junction proteins between enterocytes can be damaged. TA supplementation quadratically decreased gut permeability in broilers infected with *E. maxima*, and the TA2.75 group had significantly lower gut permeability compared to the CC group in the present study. In agreement with this, TA supplementation tended to quadratically increase relative mRNA expression of ZO2 in the current study. Along with increased mRNA expression of tight junction proteins, TA supplementation quadratically increased relative mRNA expression of IL1β and NFκB. Increased mRNA expression of IL1β and NFκB levels have been considered as negative factors for intestinal barrier integrity (Luo et al., 2012; Tan et al., 2014). However, potentially pro-inflammatory cytokines are still important in controlling several pathogens by activating the immune system and maintaining

gut barrier integrity as defensive mechanisms in broilers infected with *Eimeria* spp. (Memon et al., 2021). However, broilers fed 5000 mg/kg TA (TA5) increased gut permeability compared to the broilers fed 2750 mg/kg TA (TA2.75) in the present study. This may have been because high concentrations of TA can show cytotoxicity and impair tight junction proteins. These results indicate that TA supplementation at appropriate concentrations improves gut barrier integrity via modulating mRNA expression of tight junction proteins and stimulating the immune system of broilers infected with *E. maxima*.

In the recovery phase, AID of DM, OM, and ash were significantly increased in the CC group compared to the SCC group, which supports the fact that there was a compensatory growth in the recovery phase after mild infection of *E. maxima* in the study. The AID of DM and CP were linearly reduced and quadratically increased due to TA supplementation in the present study. It is well known that TA decreases nutrient digestibility by compromising gut health and forming a complex with proteins in monogastric animals (Koo and Nyachoti, 2019). However, the TA2.75 group had the higher AID of DM and OM compared to the CC group, indicating that TA at appropriate concentrations has the potential to increase nutrient digestibility in broilers, depending on the conditions.

The GSH is the major endogenous antioxidant in the cells of broilers (Choi et al., 2020a). A decrease in total GSH, reduced GSH, and GSH/GSSG and an increase in GSSG in the jejunum and liver by TA supplementation in the current study indicates that TA can impair the endogenous antioxidant system and induce oxidative stress in broilers infected with *E. maxima*. However, the TA0.5 group had numerically similar or even better values in GSH, GSSG, reduced GSH, and reduced GSH/GSSG compared to the CC group. Many studies showed that tannins have antioxidant capacity in animals (Jobe et al., 2019; Peng et al., 2022). However,

our current study showed that cytotoxic and proteolytic effects of TA at high concentrations can induce oxidative stress and impair the endogenous antioxidant system in broilers infected with *E. maxima* (Marzo et al., 2002).

In the current study, *E. maxima* infection reduced jejunal VH at 6 and 13 dpi in broilers, indicating restricted nutrient absorption and digestion, which is in agreement with our previous study (Teng et al., 2021a). TA supplementation quadratically increased CD of broilers infected with *E. maxima* at 6 dpi; however, this did not lead to changes in the VH and VH/CD ratio in the current study. At 13 dpi, TA supplementation linearly increased jejunal VH in the current study, which potentially explains the increased nutrient digestibility. Wang et al. (2019) reported that TA supplementation increased jejunal development in mice challenged with diquat (an oxidative stress model). The differences were due to different animals, challenge models (parasitic infection v. oxidative stress model), and ways to provide TA (feeding v. oral gavage). Broilers infected with *E. maxima* had increased goblet cell density in the present study. Goblet cells, which produce mucus, play important roles in cytoprotective functions against colonization of pathogens in the epithelium of the small intestine (Dao and Le, 2021). However, over-produced mucus can increase bacterial pathogenesis because some pathogens can use mucus as their food and habitat (Edelman et al., 2003; Wadolkowski et al., 1988). Collier et al. (2008) reported that *E. maxima* caused a host mucogenic response, which can make the intestine susceptible to be infected with pathogens (e.g., *Clostridium perfringens*) that utilize mucus for their growth and proliferation in the intestine of broilers. However, TA supplementation did not modulate the concentrations of goblet cells in the villus and crypts in the present study.

6.6. CONCLUSION

E. maxima infection significantly decreased growth performance and impaired gut health of broilers. Whereas TA supplementation at high concentrations (5000 mg/kg) resulted in decreased growth performance, nutrient digestibility, antioxidant system, and gut barrier integrity, 500 to 2750 mg/kg of TA resulted in reduced oocyst shedding, activated immune system, enhanced gut barrier integrity, and improved intestinal morphology and nutrient digestibility in broilers infected with *E. maxima*. Therefore, TA supplementation at concentrations of 500 to 2750 mg/kg have the potential to be an anti-coccidial agent and improve gut health in broilers.

7.0. CHAPTER 7 MANUSCRIPT V

EFFECTS OF TANNIC ACID SUPPLEMENTATION ON GROWTH PERFORMANCE, GUT HEALTH, GUT MICROBIOTA, AND MEAT PRODUCTION AND QUALITY OF BROILER CHICKENS RAISED FOR D 42[1]

[1]**Choi, J.**, Liu, G., Goo, D., Wang, J., Bowker, B., Zhuang. H., and W. K. Kim. Effects of tannic acid supplementation on growth performance, gut health, gut microbiota, and meat production and quality of broiler chickens on D 42. Submitted to Frontiers in Physiology.

7.1. ABSTRACT

The purpose of the study was to investigate the effects of tannic acid (TA) supplementation on growth performance, gut health, antioxidant capacity, gut microbiota, and meat yield and quality in broilers on D 42. A total of 700 one-day-old male broiler chickens (Cobb 500) were allocated into 5 treatments with 7 replicates of 20 birds per pen. There were five treatments: 1) tannic acid 0 (TA0; basal diet without TA); 2) tannic acid 0.25 (TA0.25; basal diet + 0.25 g/kg TA); 3) tannic acid 0.5 (TA0.5; basal diet + 0.5 g/kg TA); 4) tannic acid 1 (TA1; basal diet + 1 g/kg TA); and 5) tannic acid 2 (TA2; basal diet + 2 g/kg TA). The dietary phases included starter (D 0 to 18; crumble feed), grower (D 18 to 28; pellet feed), and finisher (D 28 to 42; pellet feed), and TA treatments were applied at all three dietary phases. On D 18, TA supplementation linearly reduced body weight (BW) and average daily feed intake (ADFI) ($P < 0.05$), and on D 28, TA supplementation linearly reduced BW, average daily gain (ADG), and feed conversion ratio (FCR) ($P < 0.05$). Relative mRNA expression of genes related to mucin production (MUC2), tight junction proteins (CLDN2 and JAM2), and nutrient transporters (B0AT1 and SGLT1) was linearly increased by the TA supplementation ($P < 0.05$). TA supplementation tended to linearly increase the relative abundance of the family Enterobacteriaceae ($P = 0.08$) and quadratically increased the relative abundance of the families Lachnospiraceae and Ruminococcaceae in the cecal microbial communities ($P < 0.05$). On D 36, the ratio of the phyla Firmicutes and Bacteroidetes was quadratically reduced by TA supplementation ($P < 0.05$). On D 42, bone mineral density and the lean to fat ratio were linearly decreased by TA supplementation ($P < 0.05$). On D 43, total chilled carcass weight was linearly reduced ($P < 0.05$), and proportion of leg weight was increased by TA supplementation ($P < 0.05$). TA supplementation linearly reduced pH of the breast meat ($P <$

0.05) and linearly increased redness (a*) ($P < 0.05$). Although the TA supplementation positively influenced gut health and gut microbiota in the starter/grower phases, it negatively affected overall growth performance, bone health, and meat production in broilers on D 42.

Keywords: tannic acid, gut microbiota, gut health, nutrient digestibility, floor pen, pelleting process, meat production, and fat accumulation

7.2. INTRODUCTION

In the past, antibiotic growth promoters (AGP) have been supplemented to broiler diets to enhance growth performance and gut health and to prevent diseases in broilers (Caly et al., 2015). Due to the public concerns about the spread of antibiotic resistant bacteria and their genes, there is a global movement to implement antibiotic-free production in the poultry industry (Haque et al., 2020). However, withdrawal of AGP without appropriate alternative strategies against bacterial infections could result in reduced production efficiency and broiler health and welfare issues by inducing severe microbial infection in broilers (Cervantes, 2015). It has been essential for the poultry industry to find appropriate bioactive compounds that can improve growth performance and gut health in antibiotic-free production. Diverse bioactive compounds, such as essential oils (Yang et al., 2021), amino acids (Teng et al., 2021b), organic acids (Adil et al., 2010), plant extracts (Mogire et al., 2021; Yadav et al., 2022b), and exogenous enzymes (Lu et al., 2020a), have been studied and used as AGP alternatives in poultry production. Alternatives for AGP should be able to enhance growth performance, gut health, and meat production and quality of broilers and should be safe to the public and eco-friendly and cost-effective in broiler production (Yang et al., 2015b).

Tannins, polyphenol compounds that can precipitate proteins, are considered as AGP alternatives in broiler production due to their effective antimicrobial, antioxidative, and anti-inflammatory effects in chickens (Choi and Kim, 2020). Tannic acid (TA), which is composed of 7 to 8 gallic acids molecules and one glucose molecule as a central core, is a standard of hydrolysable tannins and present in woods such as oak, chestnut, and acacia (Romani et al., 2006). Traditionally, TA was considered as an anti-nutritional factor due to its protein precipitation capacity, which can result in reduced nutrient digestibility and proteolytic activity

in the liver of chickens (Marzo et al., 2002; Redondo et al., 2014a). Many recent studies showed that the TA supplementation at appropriate dosages (up to 2 g/kg) improved growth performance, gut health, immune system, and gut microbiota in broilers under non-challenge conditions and diverse challenging conditions (*Eimeria* spp., *Salmonella* spp., etc) (Diaz Carrasco et al., 2018; Ramah et al., 2020; Tonda et al., 2018). Our previous study (Choi et al., 2022c) demonstrated that 0.5 g/kg TA increased activities of endogenous antioxidant enzymes, whereas higher than 1 g/kg TA exhibited antinutritional effects in broilers on D 21. However, it is still uncertain whether the TA supplementation (up to 2 g/kg) would beneficially or negatively influence growth performance, gut health, antioxidant capacity, gut microbiota, and meat production and quality in broilers on D 42 (slaughter age). Therefore, this study was aimed to investigate the effects of TA supplementation (up to 2 g/kg TA) on growth performance, gut health, antioxidant capacity, gut microbiota, and meat yield and quality in broilers on D 42.

7.3. MATERIALS AND METHODS

7.3.1. Animals, diets, experimental design, and growth performance

The current study was reviewed and approved by the Institutional Animal Care and Use Committee at the University of Georgia, Athens, GA. A total of 700 one-day-old Cobb 500 male broiler chickens were randomly allotted to 5 treatments with 7 replicates of 20 birds per pen in a completely randomized design. The five treatments included 1) tannic acid 0 (TA0; basal diet without TA); 2) tannic acid 0.25 (TA0.25; basal diet + 0.25 g/kg TA); 3) tannic acid 0.5 (TA0.5; basal diet + 0.5 g/kg TA); 4) tannic acid 1 (TA1; basal diet + 1 g/kg TA); and 5) tannic acid 2 (TA2; basal diet + 2 g/kg TA). The TA was purchased from Sigma-Aldrich Co., (St Louis, MO). The experiment period was divided into starter (D 0 to 18; crumble feed),

grower (D 18 to D 28; pellet feed), and finisher (D 28 to 42; pellet feed) phases, and the diets were formulated to meet or exceed recommendation levels according to the Cobb 500 nutrient requirement guide (2018) (Table 1). Conditioning temperature was 80°C for the feed pelleting process. On D 28 to 35, all diets included 0.3% titanium dioxide (Acros Organics, Morris Plains, NJ) as an inert marker to determine nutrient digestibility. Birds were raised in floor pens (width: 1.52 m, length: 1.22 m, height: 0.61 m) equipped with one feeder and three drinker nipples per pen, and birds had free access to water and feed. Temperature and light were controlled in accordance with the recommendation of the Cobb 500 broiler management guide (2018). Body weight (BW) and feed disappearance were measured on D 18, 28, and 42 to calculate average daily gain (ADG), average daily feed intake (ADFI), and feed conversion ratio (FCR).

Table 7.1. Ingredients and nutrient compositions of basal diets (As-fed basis)

Items	D 0 to 18	D 18 to 28	D 28 to 42
Feed form	Crumble	Pellet	Pellet
Ingredients (kg/ton)			
Corn	614.01	672.67	690.87
Soybean meal (480 g crude protein/kg)	323.935	263.16	239.22
Dicalcium phosphate	15.164	12.622	12.82
Filler[1]	10	10	10
Soybean oil	12.433	18.58	24
Limestone	11.564	10.571	10.655
DL-Methionine 99%	2.912	2.656	2.486
L-Lysine HCl 78%	1.974	2.003	2.144
Vitamin premix[2]	2.5	2.5	2.5
Sodium chloride	3.417	3.455	3.47
L-threonine	0.791	0.475	0.538
Mineral premix[3]	0.8	0.8	0.8
Coccidiostats[4]	0.5	0.5	0.5
Total	1,000	1,000	1,000
Calculated energy and nutrient value, %			
Metabolizable energy, kcal/kg	3,000	3,100	3,150
Crude protein	20.5	18	17
SID[4] Methionine	0.598	0.54	0.51
SID[4] Total sulfur amino acids	0.88	0.8	0.76
SID[4] Lysine	1.17	1.02	0.97
SID[4] Threonine	0.78	0.66	0.63
Total calcium	0.87	0.76	0.76
Available phosphate	0.435	0.38	0.38

[1] Sand and tannic acid were included to obtain wanted tannic acid concentrations in the feed as follows: Tannic acid 0 (TA0): sand 10 g/kg + tannic acid 0 g/kg; Tannic acid 0.25 (TA0.25): sand 9.75 g/kg + tannic acid 0.25 g/kg; Tannic acid 0.5 (TA0.5): sand 9.5 g/kg + tannic acid 0.5 g/kg; Tannic acid 1 (TA1): sand 9 g/kg + tannic acid 1 g/kg; and Tannic acid 2 (TA2): sand 8 g/kg + tannic acid 2 g/kg. Tannic acid was purchased from Sigma–Aldrich (St. Louis, MO). In the finisher phase, titanium dioxide 3 g/kg (Acros Organics, Morris Plains, NJ) were included in the sand part.

[2]Vitamin mix provided the following in mg/100 g diet: thiamine-HCl, 1.5; riboflavin 1.5; nicotinic acid amide 15; folic acid 7.5; pyridoxine-HCl, 1.2; d-biotin 3; vitamin B-12 (source

concentration, 0.1%) 2; d-calcium pantothenate 4; menadione sodium bisulfite, 1.98; α-tocopherol acetate (source 500,000 IU/g), 22.8; cholecalciferol (source 5,000,000 IU/g) 0.09; retinyl palmitate (source 500,000 IU/g), 2.8; ethoxyquin, 13.34; I-inositol, 2.5; dextrose, 762.2.
[3]Mineral mix provided the following in g/100 g diet: $Ca(H_2PO_4)_2 \cdot H_2O$, 3.62; $CaCO_3$, 1.48; KH_2PO_4, 1.00; Na_2SeO_4, 0.0002; $MnSO_4 \cdot H_2O$, 0.035; $FeSO_4 \cdot 7H_2O$, 0.05; $MgSO_4 \cdot 7H_2O$, 0.62; KIO_3, 0.001; NaCl, 0.60; $CuSO_4 \cdot 5H_2O$, 0.008; $ZnCO_3$, 0.015; $CoCl_2 \cdot 6H_2O$, 0.00032; $NaMoO_4 \cdot 2H_2O$, 0.0011; KCl, 0.10; dextrose, 0.40.
[4]Coban 90, Elanco Animal Health, Greenfield, IN
[5]SID: standard ileal digestible amino acid

7.3.2. Sampling, dual-energy X-ray absorptiometry, litter ammonia, and foot pad lesion

On D 18 and 36, one bird per pen was euthanized via cervical dislocation to collect samples of liver, intestinal tissue (mid-duodenum, mid-jejunum, and mid-ileum), and cecal content. All tissue samples were washed with PBS to remove remaining digesta and blood. Samples of liver, mid-jejunum tissue, and cecal content were snap-frozen and stored at –80°C for further analyses. For intestinal morphology, mid-duodenum, mid-jejunum, and mid-ileum samples were fixed in a 10% formaldehyde solution. On D 36, four birds were euthanized, and digesta samples from 10 cm below Meckel's diverticulum to the upper 10 cm of the ileo-cecal-colic junction were collected and oven-dried at 75°C until constant weight was achieved. On D 42, one bird per pen was euthanized via cervical dislocation and scanned using dual-energy X-ray absorptiometry (DEXA, GE Healthcare, Madison, WI) to determine total tissue weight (g), bone mineral content (BMC; g), bone mineral density (BMD; g/cm^2), lean weight (g), fat weight (g), body fat percentage (%), and lean:fat (g/g). On D 42, severity of foot pad dermatitis (FPD) was measured from all birds in each pen according to Eichner et al. (2007): score 0: no lesion; score 1: FPD covers less than 25% of the food pad; score 2: FPD covers 25 to 50 % of the food pad; and score 3: FPD covers more than 50% of the food pad. Both foot pads were checked in birds, and scores from both foot pads were averaged, and FPD incidence (%) was also calculated. Ammonia concentration (mg/kg) on the litter was measured using a Chillgard® RT Refrigerant Monitor (MSA, Cranberry Township, PA) connected to a HOBO® monitoring station (Onset, Bourne, MA) according to Aston et al. (2019).

7.3.3. Apparent ileal digestibility of dry matter, organic matter, ash, crude protein, and ether extract

Oven-dried feed (75°C till constant weight; 0.5 g) and ileal digesta (0.3 g) samples were ashed at 600°C overnight, and concentrations of titanium dioxide were determined according to Short et al. (1996). The concentration of crude protein (CP) was analyzed using nitrogen combustion analyses according to AOAC International (2000) analytical method 990.03. The crude fat (CF) was determined according to AOAC International (2000) analytical method 942.05. Apparent ileal digestibility (AID) of dry matter (DM), organic matter (OM), ash, CP, and CF was calculated according to Lin and Olukosi (2021).

7.3.4. Intestinal morphology

After 72 h of fixation in the 10% formalin solution, the fixed intestine ssamples were embedded in paraffin and cut into 4 mm, and the samples were stained with hematoxylin and eosin (H&E). The images of H&E-stained slides were taken using a microscope (BZ-X810; Keyence, Osaka, Japan). Five well-shaped villus and their corresponding crypts were selected per slide, and villus height (VH) and crypt depth (CD) were measured by using ImageJ (National Institutes of Health, Bethesda, MD). The VH to CD ratio (VH:CD) was calculated for each villi and crypt.

7.3.5. Jejunal brush border digestive enzyme activities and serum alkaline phosphatase

Around 100 mg of whole-jejunal tissue samples were homogenized in 2 mL PBS using a bead beater (Biospec Products, Bartlesville, OK). Afterwards, the samples were centrifuged at 12,000 × *g* for 15 min at 4°C, and protein concentrations of the supernatants was determined using Pierce BCA protein assay kits (Thermo Fisher Scientific, Waltham, MA). Activities of maltase and sucrase in the supernatants were analyzed according to Fan et al. (2004). Activities

of lipase in the supernatants were determined according to Elgharbawy et al. (2018). Alanine-aminopeptidase (APN) activities were assayed according to Maroux et al. (1973). Activities of alkaline phosphatase in the supernatant and serum were analyzed according to Lackeyram et al. (2010). The activities of the enzymes were expressed as their values per mg protein.

7.3.6. RNA extraction and real-time reverse transcription (RT)-PCR analysis

Approximately 100 mg of the whole jejunal tissue samples were homogenized using a bead beater (Biospec Products, Bartlesville, OK) in QIAzol lysis reagents (Qiagen, Valencia, CA). Afterwards, RNA was extracted according to the manufacturer's protocol. RNA quantity and quality were measured by a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). One microgram of RNA was used to synthesize the first-strand cDNA by using high-capacity cDNA synthesis kits (Applied Biosystems, Foster City, CA). Primers used in the study are listed in Table 2. Real-time reverse transcript (RT)-PCR was conducted using SYBR Green Master Mix with a Step Onethermocycler (Applied Biosystem). The final volume for PCR mixture was 10 μL which included 5 μL of SYBR Green Master Mix (Applied Biosystems), 1.5 μL of cDNA, 0.5 μl of forward and reverse primers (10 μM), and 2.5 μL of water. The thermal cycle condition for all genes was 95°C denature for 10 min, 40 cycles at 95°C for 15 s and 60°C for 1 min, 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and beta-actin were used as reference genes to normalize target mRNA abundance (Vandesompele et al., 2002). Relative mRNA abundance of target genes was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Each sample was analyzed in duplicate, and the negative control, containing water instead of cDNA, was included in each run.

Table 7.2. Primers used in the study

Genes	Sequence, 5′ to 3′	Amplicon
GAPDH	F: GCT AAG GCT GTG GGG AAA GT R: TCA GCA GCA GCC TTC ACT AC	161
Beta actin	F: CAA CAC AGT GCT GTC TGG TGG TA R: ATC GTA CTC CTG CTT GCT GAT CC	205
ZO2	F: ATC CAA GAA GGC ACC TCA GC R: CAT CCT CCC GAA CAA TGC	100
CLDN2	F: CCT GCT CAC CCT CAT TGG AG R: GCT GAA CTC ACT CTT GGG CT	145
MUC2	F: ATG CGA TGT TAA CAC AGG ACT C R: GTG GAG CAC AGC AGA CTT TG	110
B0AT1	F: GGG TTT TGT GTT GGC TTA GGA A R: TCC ATG GCT CTG GCA GAG AT	60
PepT1	F: CCC CTG AGG AGG ATC CTT R: CAA AAG AGC AGC AAC GA	66
SGLT1	F: GCC ATG GCC AGG GCT TA R: CAA TAA CCT GAT CTG TGC ACC AGT A	71
EAAT3	F: TGC TGC TTT GGA TTC CAG TGT R: AGC AAT GAC TGT AGT GCA GAA GTA ATA TAT G	79

[1]GAPDH, glyceraldehyde 3-phosphate dehydrogenase; ZO2, zonula occludens 2; CLDN2, claudin 2; MUC2, mucin 2; B0AT1, sodium-dependent neutral amino acid transporter 1; PepT1, peptide transporter 1; SGLT1, sodium glucose transporter 1; and EAAT3, excitatory amino acid transporter 3.

7.3.7. Liver total antioxidant capacity, concentrations of glutathione and oxidized glutathione, and activities of superoxide dismutase

Approximately 100 mg of liver samples were homogenized using a bead beater (Biospec Products, Bartlesville, OK) in the selected solution for each assay. Afterwards, the samples were centrifuged at 12,000 × *g* for 15 min at 4°C, and protein concentration of the supernatants were analyzed using Pierce BCA protein assay kits (Thermo Fisher Scientific) after 20-time sample dilution. The total antioxidant capacity (TAC) of the collected supernatant was analyzed using a commercial kit (QuantiCromAntioxidant Assay Kit, BioAssay Systems, Hayward, CA) after 2-time sample dilution. Concentrations of glutathione (GSH) and oxidized glutathione (GSSG) in the supernatant were analyzed using Caymans GSH assay kits (Cayman Chemical, Ann Arbor, MI) with 20- and 2-time sample dilutions, respectively. The activities of superoxide dismutase (SOD) in the supernatants were determined Caymans SOD assay kits (Cayman Chemical) after 400-time sample dilution. The TAC, concentrations of GSH and GSSG, and SOD activities were expressed as values per mg protein.

7.3.8. DNA extraction and microbiome analysis

DNA was extracted from the cecal contents using QIAamp® DNA stool mini kits (Qiagen GmbH, Hilden, Germany) according to manufacturer's protocol. After quality and quantity of extracted DNA were checked using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific), the samples were sent to LC sciences (Houston, TX) for 16s rRNA gene sequencing (Choi et al., 2022c). Qimme2 (version 2022.02) was used to process and analyze 16s rRNA gene sequences (Bolyen et al., 2019). According to Choi et al. (2022c), 16s rRNA sequences were processed. The sampling depth for both D 18 and 36 time points was set as

45,000. By using Qiime2's built-in functions, alpha diversity, beta diversity, and phylum and family level composition were analyzed and presented.

7.3.9. Slaughter, meat processing, and breast myopathies

On D 42, three birds per pen were randomly selected from each pen for processing, and feed was removed from the pen for 12 h (Wang et al., 2020a). On D 43, the selected birds were individually weighed and transferred to the processing plant at the University of Georgia. Birds were shackled, electrically stunned, bled, scalded, and defeathered. Following head and feet removal, the carcasses were eviscerated. Weights of the hot carcass and abdominal fat collected from fat around cloaca, bursa of Fabricius, gizzard, and proventriculus (Castro et al., 2019) were recorded. The carcasses were rinsed and chilled in ice-cold water at 1°C for 4 h. Legs, breast muscle, tender, wings, and skeleton were separated by trained personnel, and their weights were recorded. Breast myopathies and quality defects including white striping [score 0 (normal), 1, 2, and 3 (severe)], woody breast [score 1 (normal), 2, and 3 (severe)], spaghetti meat [score 0 (normal), 1, and 2 (severe)], and petechial hemorrhagic lesions [score 0 (normal), 1, 2, and 3 (severe)] were assessed by a trained expert according to published criteria (Baldi et al., 2021; Kuttappan et al., 2017; Pang et al., 2020; Prisco et al., 2021).

7.3.10. Color, pH, drip loss, thawing loss, and cooking loss of breast meat

Breast muscles from two birds per pen were stored at 1°C overnight for further meat quality analyses. Color and pH of the breast muscles were analyzed according to Brambila et al. (2018) with the modification. Color indicators including lightness (L*), redness (a*), and yellowness (b*) were determined in duplicate on the dorsal surface of breast meat by a Minolta Spectrophotometer CM-700d (Konica Minolta Inc., Ramsey, NJ). Meat pH was analyzed (one measurement per fillet) by using a Thermo Scientific Orion Star^{TM} A221 portable pH meter

with a spear tipped probe (Thermal Scientific Orion 8163BNWP) (Thermo Fisher Scientific, Waltham, MA 02451, USA) that penetrated the cranial end of the intact breast muscle. Drip loss was analyzed by using a EZ-driploss method (Kaić et al., 2021). One cylindrical muscle core (2.5-cm diameter) was removed from the cranial side of the breast meat and trimmed to a similar height. The cores were weighed and placed in individual EZ containers (Danish Meat Research Institute, Taastrup, Denmark). The sealed containers were then stored in a refrigerator at 4°C. The samples were reweighted (approximately 7 to 8 g) after 48 h to determine drip loss (%). For thawing loss (%), intact breast samples were weighed and individually sealed in cooking bags before frozen at −20°C. The frozen samples were thawed at 4°C overnight and weighed again after liquid was removed. For cooking loss, the intact breast samples packed in cooking bags were cooked in a combi oven to the endpoint temperature of 74°C.The samples were reweighed after the liquid was removed (Brambila et al., 2018).

7.3.11. Statistical analyses

SAS (version 9.4; SAS Inst. Inc., Cary, NC) and GraphPad Prism (Version 9.1.0; GraphPad Software, San Diego, CA) were used for statistical analyses and graph construction. Treatment groups were compared using PROC MIXED followed by the Tukey's individual comparison test. Orthogonal polynomial contrasts were conducted to assess the significance of linear or quadratic effects of the TA supplementation in broilers. Breast meat myopathy score and FPD score and incidence were analyzed using the Kruskal–Wallis test followed by the Dwass–Steel–Critchlow–Fligner post hoc test. Significance level was set at $P < 0.05$, and tendencies were also presented at $0.05 < P \leq 0.10$ (Choi et al., 2021).

7.4. RESULTS

7.4.1. Growth performance

Results of the growth performance are presented in Table 7.3. In the starter phase, the TA2 group had significantly lower BW and ADG compared to the TA0 group, and the TA supplementation linearly decreased BW and ADG in broiler chickens ($P < 0.01$). The ADFI was also linearly reduced by the TA supplementation in broilers in the starter phase ($P < 0.05$). In the grower phase, the TA supplementation linearly reduced BW and ADG and linearly and quadratically increased FCR of broilers ($P < 0.01$). The TA2 group had significantly lower BW compared to the TA0 group. The TA2 group had significantly lower ADG compared to the TA0 and TA1 groups ($P < 0.05$). The TA0.5 group had significantly lower ADG compared to the TA0 group and had significantly lower ADG compared to the TA1 group. The TA2 group had the highest FCR ($P < 0.05$) among the treatment groups, and the TA1 group had significantly lower FCR compared to the TA0.5 group. No statistical differences were observed in the growth performance parameters of the finisher phase ($P > 0.1$). In the whole phase, the TA supplementation linearly increased FCR ($P < 0.01$), and the TA2 group had significantly higher FCR compared to the TA0 group.

7.4.2. Intestinal morphology

As shown in Table 7.4., the TA0 group had significantly higher jejunal CD compared to the TA supplemented group, and the TA supplementation linearly ($P < 0.01$) and quadratically ($P < 0.05$) reduced jejunal CD on D 18. The TA0.5 and TA2 groups had greater jejunal VH:CD compared to the TA0 group ($P < 0.05$), and the TA supplementation linearly increased jejunal VH:CD. The TA supplementation tended to linearly decrease ileal VH ($P = 0.075$). The TA0.25 tended to have lower ileal CD compared to the TA0 group ($P = 0.057$).

The TA supplementation quadratically decreased ileal CD and increased ileal VH:CD ($P < 0.05$). There were no statistical differences in the intestinal morphology on D 36 ($P > 0.1$).

Table 7.3. Growth performance parameters including body weight (BW, g), average daily gain (ADG, g/d), average daily feed intake (ADFI, g/d), and feed conversion ratio (FCR, g/g) in broilers fed diets supplemented with tannic acid on D 42[1]

								Polynomial contrast[3]	
Items	TA0	TA0.25	TA0.5	TA1	TA2	SEM	*P* value[2]	Linear	Quadratic
Initial BW, g	45.96	45.94	45.94	45.96	45.94	0.09	0.986		
Starter (D 0 to 18)									
BW	890.24^{a}	860.97^{ab}	866.14^{ab}	853.03^{ab}	822.86^{b}	38.63	0.045	0.003	0.787
ADG	46.90^{a}	45.28^{ab}	45.57^{ab}	44.84^{ab}	43.16^{b}	2.14	0.044	0.003	0.787
ADFI	59.79	58.09	58.39	57.27	56.64	2.47	0.187	0.030	0.411
FCR	1.28	1.28	1.28	1.28	1.31	0.04	0.469	0.108	0.479
Grower (D 18 to 28)									
BW	2020.4^{a}	1959.23^{ab}	1943.7^{ab}	1973.35^{ab}	1892.55^{b}	56.15	0.004	0.001	0.911
ADG	113.19^{a}	109.65^{abc}	107.76^{bc}	112.03^{ab}	106.97^{c}	2.89	0.001	0.007	0.948
ADFI	173.42	170.73	168.97	170.98	173.66	4.64	0.305	0.445	0.089
FCR	1.53^{bc}	1.56^{bc}	1.57^{b}	1.53^{c}	1.62^{a}	0.03	<0.001	<0.001	0.005
Finisher (D 28 to 42)									
BW	3772.68	3671.24	3630.39	3711.05	3634.49	116.86	0.154	0.155	0.528
ADG	125.16	122.29	120.48	124.12	124.42	6.35	0.648	0.709	0.447
ADFI	214.79	212.52	214.21	216.75	218.59	10.86	0.857	0.323	0.927
FCR	1.72	1.74	1.78	1.75	1.76	0.07	0.616	0.507	0.413
Whole (D 0 to 42)									
ADG	88.77	86.28	85.34	87.26	85.44	2.77	0.143	0.152	0.514
ADFI	138.51	136.38	136.66	137.5	138.49	4.42	0.839	0.642	0.481
FCR	1.48^{b}	1.50^{ab}	1.52^{ab}	1.49^{ab}	1.53^{a}	0.03	0.023	0.008	0.713

[1]TA0 (tannic acid 0; basal diet); TA0.25 (tannic acid 0.25; basal diet + 0.25 g/kg TA); TA0.5 (tannic acid 0.5; basal diet + 0.5 g/kg TA); TA1 (tannic acid 1; basal diet + 1 g/kg TA); and TA2 (tannic acid 2; basal diet + 2 g/kg TA).

[2]Treatment groups were compared using PROC MIXED followed by the Tukey's individual comparison test. Different letters in the same row means significant differences ($P < 0.05$) among treatments.

[3]Orthogonal polynomial contrasts were conducted to assess the significance of linear or quadratic effects of TA supplementation in broilers.

Table 7.4. Duodenal, jejunal, and ileal morphology parameters including villus height (VH, μm), crypt depth (CD, μm), and VH:CD in in broilers fed diets supplemented with tannic acid on D 18 and 36[1]

								Polynomial contrast[3]	
Items	TA0	TA0.25	TA0.5	TA1	TA2	SEM	*P* value[2]	Linear	Quadratic
D 18									
Duodenal VH	2239.4	2282.5	2337.6	2256.7	2269.6	260.8	0.964	0.979	0.760
Duodenal CD	212	221.4	215.9	214.0	191	39.71	0.664	0.195	0.478
Duodenal VH:CD	11.23	11.18	11.08	10.69	12.21	1.86	0.637	0.305	0.268
Jejunal VH	1240.7	1151.5	1218.1	1205.7	1198.8	194.83	0.937	0.926	0.890
Jejunal CD	286.36[a]	219.76[b]	203.72[b]	223.19[b]	200.78[b]	36.08	<0.001	0.003	0.027
Jejunal VH:CD	4.55[b]	5.52[ab]	6.29[a]	5.59[ab]	6.26[a]	1.09	0.036	0.031	0.209
Ileal VH	916.94	805.3	910.39	856.22	791.58	103.75	0.096	0.075	0.745
Ileal CD	222.44[a]	173.9[b]	185.65[ab]	184.37[ab]	193.79[ab]	30.29	0.057	0.517	0.046
Ileal VH:CD	4.26	4.88	5.10	4.85	4.26	0.815	0.212	0.451	0.046
D 36									
Duodenal VH	2596.9	2718.2	2582.5	2608.2	2849.4	424.4	0.737	0.311	0.522
Duodenal CD	206.66	201.69	172.23	198.67	204.99	33.85	0.325	0.731	0.224
Duodenal VH:CD	13.09	14.62	15.91	13.70	14.56	2.93	0.459	0.768	0.536
Jejunal VH	1543.5	1781.9	1410.2	1795.8	1758	248.96	0.042	0.109	0.796
Jejunal CD	179.9	186.74	163.79	175.59	193.21	28.03	0.366	0.342	0.201
Jejunal VH:CD	9.11	10	8.89	11.24	9.4	1.88	0.165	0.661	0.113
Ileal VH	1050.5	912.5	958.5	1010.1	1016.7	147.93	0.454	0.657	0.496
Ileal CD	157.47	155.29	149.88	149.77	157.91	24.36	0.943	0.912	0.412
Ileal VH:CD	6.96	6.32	6.78	7.04	6.68	1.28	0.849	0.971	0.795

[1]TA0 (tannic acid 0; basal diet); TA0.25 (tannic acid 0.25; basal diet + 0.25 g/kg TA); TA0.5 (tannic acid 0.5; basal diet + 0.5 g/kg TA); TA1 (tannic acid 1; basal diet + 1 g/kg TA); and TA2 (tannic acid 2; basal diet + 2 g/kg TA).

[2]Treatment groups were compared using PROC MIXED followed by the Tukey's individual comparison test. Different letters in the same row means significant differences ($P < 0.05$) among treatments.

[3]Orthogonal polynomial contrasts were conducted to assess the significance of linear or quadratic effects of TA supplementation in broilers.

7.4.3. Activities of jejunal brush border digestive enzymes and serum alkaline phosphatase

As shown in Table 7.5., the TA supplementation quadratically increased sucrase activities ($P < 0.05$) and quadratically decreased lipase activities in the jejunum tissue ($P < 0.05$). On D 36, jejunal lipase activities were quadratically decreased by the TA supplementation ($P < 0.05$). However, no differences were observed in the activities of jejunal sucrase, APN, intestinal alkaline phosphatase (IAP), serum alkaline phosphatase (SAP) ($P > 0.1$).

7.4.4. Apparent ileal digestibility of dry matter, organic matter, ash, crude protein, and ether extract

As shown in Table 7.6, the TA0.25, TA0.5, TA1, and TA2 groups had significantly higher AID of DM compared to the TA0 group ($P < 0.01$), and the TA supplementation linearly ($P < 0.01$) and quadratically ($P < 0.05$) increased AID of DM. The AID of OM was significantly lower in the TA0 group compared to the TA2 group. The TA0 group had the lowest AID of CP among the treatments ($P < 0.05$). No differences were observed in the AID of CF among the treatments.

Table 7.5. Activities of jejunal brush border digestive enzymes including maltase (nmol glucose released/mg protein/min), sucrase (nmol glucose released/mg protein/min), aminopeptidase N (nmol p-nitroaniline liberated/mg protein/min), intestinal alkaline phosphatase (IAP; μmol p-nitrophenol liberated/mg protein/min), lipase (mmol p-nitrophenyl phosphate liberated/mg protein/min), and serum alkaline phosphatase (SAP; μmol p-nitrophenol liberated/mL serum/min) in broilers fed diets supplemented with tannic acid on D 18 and 36[1]

								Polynomial contrast[3]	
Items	TA0	TA0.25	TA0.5	TA1	TA2	SEM	[2]*P* value	Linear	Quadratic
D 18									
Maltase	0.218	0.247	0.227	0.233	0.232	0.066	0.951	0.906	0.826
Sucrase	0.162	0.277	0.334	0.315	0.243	0.136	0.170	0.634	0.027
APN	25.41	25.88	20.55	25.84	22.27	6.23	0.388	0.452	0.968
Lipase	1.612	1.138	0.751	0.861	1.212	0.623	0.113	0.533	0.016
IAP	0.206	0.241	0.196	0.213	0.219	0.053	0.596	0.912	0.775
SAP	0.23	0.26	0.25	0.23	0.26	0.06	0.844	0.634	0.791
D 36									
Maltase	2.221	1.809	1.876	1.750	1.815	0.681	0.713	0.421	0.353
Sucrase	0.453	0.497	0.426	0.326	0.408	0.209	0.639	0.417	0.350
APN	12.77	13.67	11.37	11.64	12.71	2.65	0.495	0.775	0.236
Lipase	1.250	1.148	1.115	0.996	1.244	0.249	0.316	0.930	0.038
IAP	0.254	0.238	0.235	0.256	0.271	0.044	0.544	0.199	0.492
SAP	0.21	0.20	0.20	0.18	0.2	0.04	0.680	0.442	0.226

[1]TA0 (tannic acid 0; basal diet); TA0.25 (tannic acid 0.25; basal diet + 0.25 g/kg TA); TA0.5 (tannic acid 0.5; basal diet + 0.5 g/kg TA); TA1 (tannic acid 1; basal diet + 1 g/kg TA); and TA2 (tannic acid 2; basal diet + 2 g/kg TA).

[2]Treatment groups were compared using PROC MIXED followed by the Tukey's individual comparison test.

[3]Orthogonal polynomial contrasts were conducted to assess the significance of linear or quadratic effects of TA supplementation in broilers.

Table 7.6. Apparent ileal digestibility (%) of dry matter (DM), organic matter (DM), organic matter (OM), ash, crude protein (CP), and ether extract (EE) in broilers fed diets supplemented with tannic acid on D 18 and 36[1]

								Polynomial contrast3	
Items	TA0	TA0.25	TA0.5	TA1	TA2	SEM	*P* value[2]	Linear	Quadratic
DM	74.37^{b}	81.00^{a}	79.26^{a}	79.24^{a}	80.68^{a}	2.39	<0.01	0.003	0.033
OM	40.39^{b}	49.57^{ab}	48.86^{ab}	50.60^{ab}	52.79^{a}	6.75	0.194	0.008	0.115
Ash	76.10^{b}	82.65^{a}	80.83^{a}	80.71^{a}	82.12^{a}	2.22	<0.01	0.003	0.03
CP	80.57^{b}	87.35^{a}	85.08^{a}	85.19^{a}	86.62^{a}	2.09	<0.01	0.002	0.028
EE	84.62	90.41	85.82	85.98	87.01	4.52	0.185	0.962	0.948

[1]TA0 (tannic acid 0; basal diet); TA0.25 (tannic acid 0.25; basal diet + 0.25 g/kg TA); TA0.5 (tannic acid 0.5; basal diet + 0.5 g/kg TA); TA1 (tannic acid 1; basal diet + 1 g/kg TA); and TA2 (tannic acid 2; basal diet + 2 g/kg TA).

[2]Treatment groups were compared using PROC MIXED followed by the Tukey's individual comparison test. Different letters in the same row means significant differences ($P < 0.05$) among the treatments.

[3]Orthogonal polynomial contrasts were conducted to assess the significance of linear or quadratic effects of TA supplementation in broilers.

7.4.5. Relative mRNA expression of genes related to tight junction proteins and nutrient transporters

Relative mRNA expression of genes related to tight junction proteins and nutrient transporters in the jejunum is presented in Table 7.7. On D 18, the TA1 group had significantly higher relative mRNA expression of zonula occludens 2 (ZO2) compared to the TA0, TA0.25 and TA0.5 groups, and the TA supplementation quadratically increased relative mRNA expression of ZO2 (P <0.05). The TA supplementation linearly increased relative mRNA expression of claudin 2 (CLDN2; $P < 0.01$) and junctional adhesion molecule 2 (JAM2; $P < 0.05$). The TA2 group had significantly higher relative mRNA expression of mucin 2 (MUC2) compared to the TA0.25 group ($P < 0.05$), and the TA supplementation linearly increase relative mRNA expression of MUC2 ($P < 0.05$). The TA1 group had significantly higher relative mRNA expression of sodium-dependent neutral amino acid transporter (B0AT1) compared to the TA0.25 group ($P < 0.05$), and the TA supplementation linearly increase relative mRNA expression of B0AT1 ($P < 0.05$). The TA1 and TA2 groups had significantly higher relative mRNA expression of sodium glucose cotransporter 1 (SGLT1) compared to the TA0.25 group, and the TA supplementation linearly increased relative mRNA expression of SGLT1 ($P < 0.01$). The TA1 group had significantly higher relative mRNA expression of excitatory amino acid transporter 3 (EAAT3) compared to the TA0, TA0.25, and TA0.5 groups, and the TA supplementation quadratically increased relative mRNA expression of EAAT3 ($P < 0.05$). However, no differences were observed among the treatments on D 36 ($P > 0.1$).

Table 7.7. Relative mRNA expression of gene associated with tight junction proteins and nutrients transporters in broilers fed diets supplemented with tannic acid on D 18 and 36[1]

								Polynomial contrast	
Items	TA0	TA0.25	TA0.5	TA1	TA2	SEM	*P* value	Linear	Quadratic
D 18									
ZO2	1.11[b]	1.11[b]	1.14[b]	1.93[a]	1.31[ab]	0.51	0.021	0.148	0.022
CLDN2	1.08	0.87	1.34	1.59	2.09	0.86	0.103	0.009	0.94
JAM2	1.42	0.84	0.72	1.03	4.6	3.23	0.16	0.035	0.171
MUC2	1.16[ab]	0.76[b]	1.01[ab]	1.47[ab]	1.79[a]	0.56	0.017	0.003	0.67
B0AT1	1.06[ab]	0.54[b]	0.91[ab]	1.84[a]	1.38[ab]	0.69	0.017	0.039	0.195
PepT1	1.12	0.9	1.29	2.03	1.32	0.71	0.061	0.203	0.038
SGLT1	1.14[ab]	0.77[b]	0.93[ab]	1.65[a]	1.76[a]	0.54	0.005	0.001	0.894
EAAT3	1.11	1.11[b]	1.14[b]	1.93[a]	1.31[ab]	0.51	0.022	0.148	0.022
D 36									
ZO2	1.14	2.03	1.49	1.7	1.48	1.04	0.606	0.95	0.451
CLDN2	1.84	1.55	1.26	0.98	1.51	1.84	0.926	0.762	0.385
JAM2	1.59	1.44	5.44	2.76	2.67	3.18	0.158	0.692	0.187
MUC2	1.02	1.12	1.56	1.83	1.43	0.53	0.064	0.116	0.015
B0AT1	1.09	0.97	1.07	1.54	1.18	0.71	0.233	0.313	0.17
PepT1	1.59	0.8	1.78	1.38	1.45	1.4	0.748	0.885	0.979
SGLT1	1.1	1.21	1.49	1.69	1.55	0.59	0.331	0.130	0.146
EAAT3	2.3	1.8	1.93	1.02	1.19	2	0.741	0.262	0.524

[1]TA0 (tannic acid 0; basal diet); TA0.25 (tannic acid 0.25; basal diet + 0.25 g/kg TA); TA0.5 (tannic acid 0.5; basal diet + 0.5 g/kg TA); TA1 (tannic acid 1; basal diet + 1 g/kg TA); and TA2 (tannic acid 2; basal diet + 2 g/kg TA). ZO2, zonula occludens 2; CLDN2, claudin 2; MUC2, mucin 2; B0AT1, sodium-dependent neutral amino acid transporter 1; PepT1, peptide transporter 1; SGLT1, sodium glucose transporter 1; and EAAT3, excitatory amino acid transporter 3

[2]Treatment groups were compared using PROC MIXED followed by the Tukey's individual comparison test. Different letters in the same row means significant differences ($P < 0.05$) among the treatments.

[3]Orthogonal polynomial contrasts were conducted to assess the significance of linear or quadratic effects of TA supplementation in broilers.

7.4.5. Liver total antioxidant capacity, concentrations of glutathione and oxidized glutathione, and activities of superoxide dismutase

No differences were observed in TAC, concentrations of GSH and GSSG, activities of SOD in the liver on D 18 and 36 (Table 7.8; $P > 0.1$).

7.4.6. Alpha diversity in the cecal bacterial communities

Alpha diversity indices including faith's phylogenetic diversity, observed features, pielou evenness, and shannon entropy in the cecal bacterial communities on D 18 and 36 are shown in Figure 7.1. On D 18, the TA1 and TA2 groups had significantly lower faith's phylogenetic diversity (communities' evolutionary distance) compared to the TA0 group ($P < 0.05$), and the TA supplementation linearly reduced faith's phylogenetic diversity in the cecal bacterial communities ($P < 0.05$). On D 36, the TA2 group had lower faith's phylogenetic diversity and observed features (richnesss) compared to TA0, TA0.25, and TA0.5 groups, and faith's phylogenetic diversity and observed features were linearly reduced by the TA supplementation ($P < 0.01$).

Table 7.8. Total antioxidant capacity (TAC; μM Trolox Equivalents/mg protein), concentrations of glutathione (GSH; μM/mg protein) and oxidized glutathione (GSSG; μM/mg protein), and activities of superoxide dismutase (SOD; U/mg protein) in broilers fed diets supplemented with tannic acid on D 18 and 36[1]

								Polynomial contrast	
Items	TA0	TA0.25	TA0.5	TA1	TA2	SEM	*P* value	Linear	Quadratic
D 18									
TAC	78.90	80.51	76.48	80.28	78.23	4.29	0.923	0.898	0.929
GSH	15.36	17.17	14.14	13.69	15.16	3.06	0.271	0.491	0.210
GSSG	2.07	2.25	1.8	1.86	1.96	0.51	0.509	0.494	0.337
Reduced GSH	11.22	12.67	10.54	9.97	11.24	2.17	0.219	0.517	0.188
Reduced GSH: GSSG	5.64	5.67	6.05	5.37	5.93	0.92	0.676	0.753	0.650
SOD	23.04	22.27	20.91	23.7	22.7	6.93	0.958	0.874	0.964
D 36									
TAC	90.31	80.65	77.94	81.93	79.12	8.38	0.073	0.114	0.146
GSH	17.11	15.83	14.37	16.20	15.96	4.3	0.828	0.896	0.543
GSSG	1.87	1.84	1.59	1.79	1.80	0.59	0.931	0.954	0.636
Reduced GSH	12.91	12.14	11.21	12.61	11.58	3.33	0.891	0.667	0.881
Reduced GSH: GSSG	7.01	7.02	7.16	7.25	6.54	1.33	0.902	0.512	0.461
SOD	24.34	25.57	25.51	25.24	25.5	8.29	0.998	0.879	0.883

[1]TA0 (tannic acid 0; basal diet); TA0.25 (tannic acid 0.25; basal diet + 0.25 g/kg TA); TA0.5 (tannic acid 0.5; basal diet + 0.5 g/kg TA); TA1 (tannic acid 1; basal diet + 1 g/kg TA); and TA2 (tannic acid 2; basal diet + 2 g/kg TA).

[2]Treatment groups were compared using PROC MIXED followed by the Tukey's individual comparison test. Different letters in the same row means significant differences ($P < 0.05$) among the treatments.

[3]Orthogonal polynomial contrasts were conducted to assess the significance of linear or quadratic effects of TA supplementation in broilers.

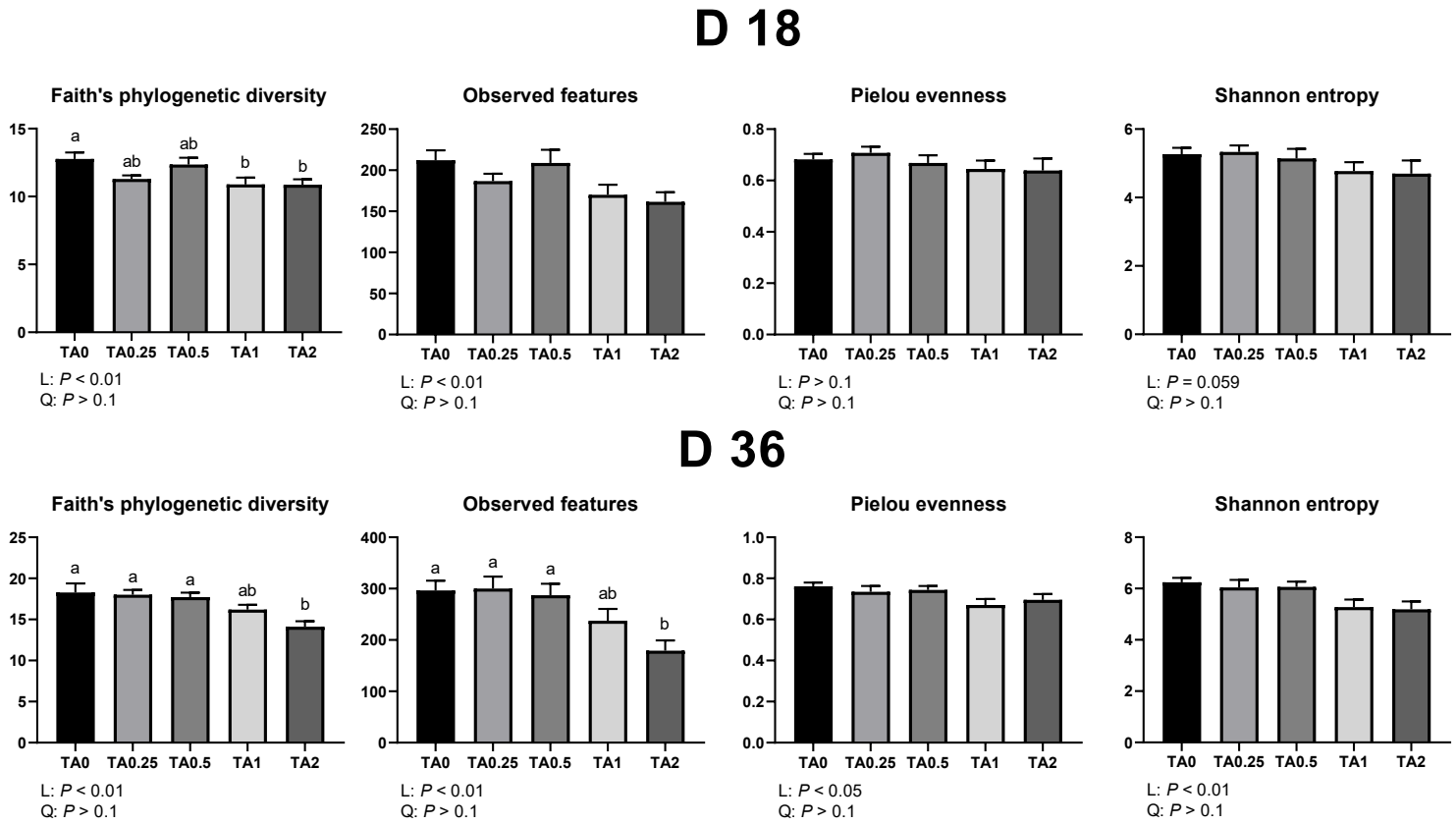

Figure 7.1. Alpha diversity parameters of the cecal microbial communities in the TA0 (tannic acid 0; basal diet), TA0.25 (tannic acid 0.25; basal diet + 0.25 g/kg TA), TA0.5 (tannic acid 0.5; basal diet + 0.5 g/kg TA), TA1 (tannic acid 1; basal diet + 1 g/kg TA), and TA2 (tannic acid 2; basal diet + 2 g/kg TA) groups on D 18 and 36. All treatment groups were compared using PROC MIXED followed

by the Tukey's individual comparison test. Different letters in the same row means significant differences ($P < 0.05$) among the treatments. Orthogonal polynomial contrasts were used to evaluate the significance of linear or quadratic effects of TA supplementation in broilers.

7.4.7. Beta diversity in the cecal bacterial communities

As shown in Figure 7.2., the TA1 group had significantly greater unweighted unifrac distance (the sum of the branch length without considering bacterial abundance) compared to the TA0 group on D 18. The TA0.5, TA1, and TA2 groups had significantly greater unweighted unifrac distance compared to the TA0.25 group. The TA0.25 group had significantly greater unweighted unifrac distance compared to the TA0.5 group ($P < 0.05$). The TA0.5 and TA2 groups had significantly greater weighted unifrac distance (the sum of the branch length with considering bacterial abundance) compared to the TA0 group. The TA0.5 and TA2 groups had significantly greater weighted unifrac distance compared to the TA0.25 group. On D 36, TA0.25 had significantly lower unweighted unifrac distance compared to the TA0 group. The TA2 group had significantly greater unweighted unifrac distance compared to the TA0.25 and TA0.5 groups. The TA0, TA0.25, and TA0.5 groups had significantly higher unweighted unifrac distance compared to the TA2 group. The TA1 and TA2 groups had significantly greater weighted unifrac distance compared to the TA0 group. The TA2 group had significantly higher weighted unifrac distance compared to the TA0.25 group. However, no visual differences were observed in the beta diversity indices including weighted and unweighted emperor on D 18 and 36 (Figure 7.3.).

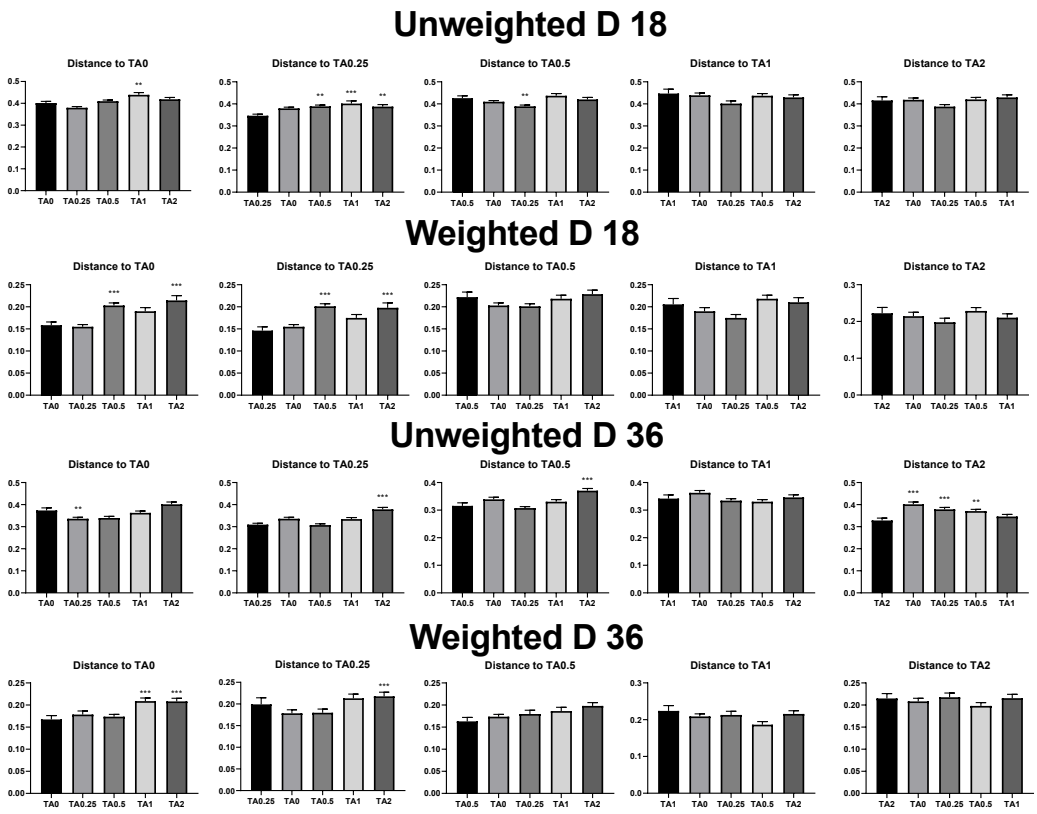

Figure 7.2. Beta diversity indices including unweighted and weighted unifrac of the cecal microbial communities in the TA0 (tannic acid 0; basal diet), TA0.25 (tannic acid 0.25; basal diet + 0.25 g/kg TA), TA0.5 (tannic acid 0.5; basal diet + 0.5 g/kg TA), TA1 (tannic acid 1; basal diet + 1 g/kg TA), and TA2 (tannic acid 2; basal diet + 2 g/kg TA) groups on D 18 and 36. All treatment groups were compared using PROC MIXED followed by the Tukey's individual comparison test. Orthogonal polynomial contrasts were used to evaluate the significance of linear or quadratic effects of TA supplementation in broilers.

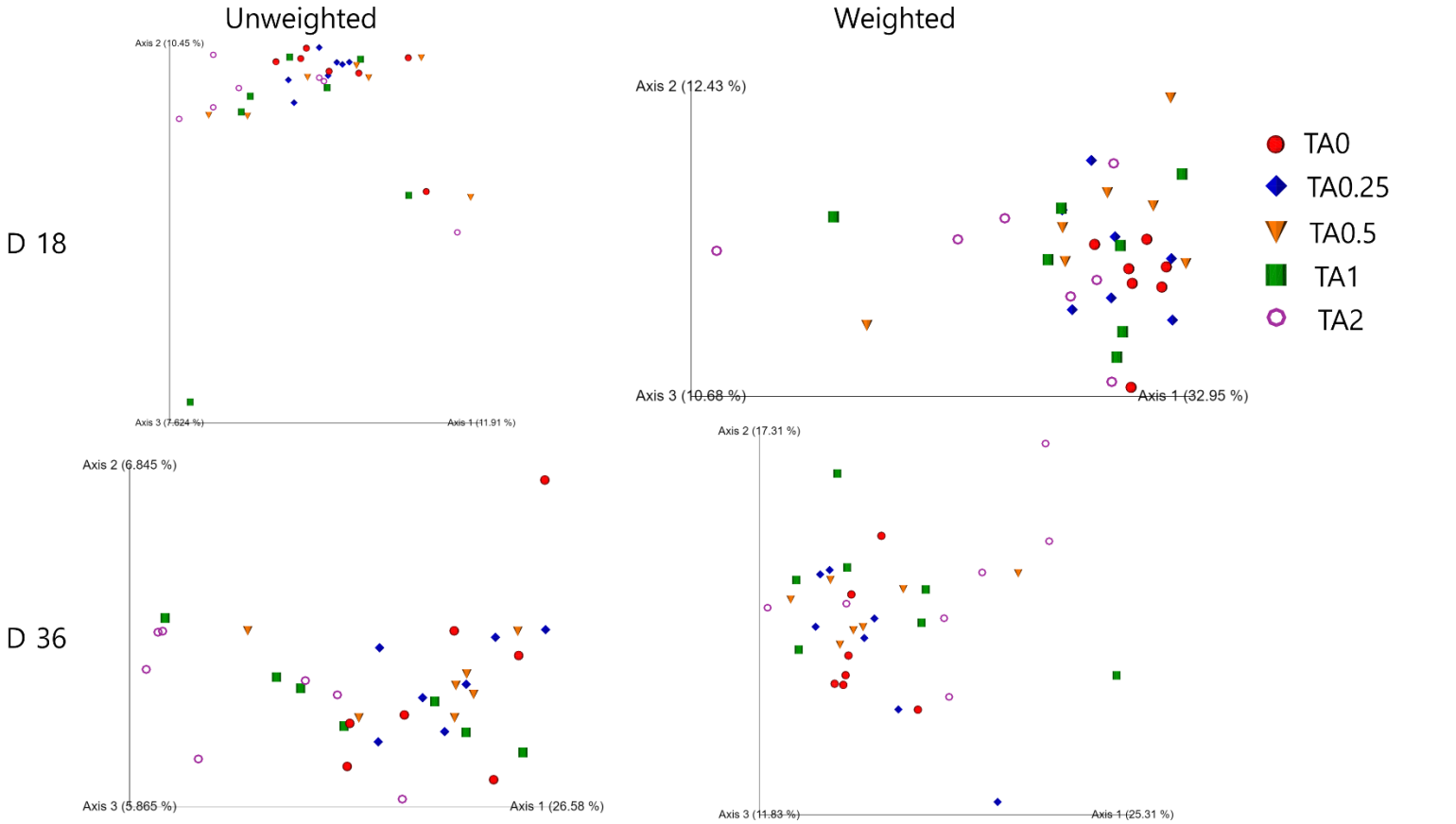

Figure 7.3. Visualized beta diversity parameters including unweighted and weighted unifrac of the cecal microbial communities in the TA0 (tannic acid 0; basal diet), TA0.25 (tannic acid 0.25; basal diet + 0.25 g/kg TA), TA0.5 (tannic acid 0.5; basal diet + 0.5 g/kg TA), TA1 (tannic acid 1; basal diet + 1 g/kg TA), and TA2 (tannic acid 2; basal diet + 2 g/kg TA) groups on D 18 and 36. All treatment

groups were compared using PROC MIXED followed by the Tukey's individual comparison test. Orthogonal polynomial contrasts were used to evaluate the significance of linear or quadratic effects of TA supplementation in broilers.

7.4.8. Bacterial composition in the cecal bacterial communities

As shown in Figure 7.4., the relative abundance of the phylum Actinobacteria was linearly increased by the TA supplementation on D 18. On D 36, the relative abundance of the phylum Firmicutes was quadratically reduced by the TA supplementation, and the TA1 group had significantly lower relative abundance of the phylum Firmicutes compared to the TA0 group. The relative abundance of the phylum Bacteroidetes was linearly ($P < 0.05$) and quadratically ($P < 0.05$) increased by the TA supplementation. The TA supplementation quadratically decreased the ratio of the phyla Firmicutes and Bacteroidetes ($P < 0.05$).

As shown in Figure 7.5., the TA supplementation tended to linearly reduce the relative abundance of the family Enterobacteriaceae ($P = 0.068$) and tended to linearly increase the relative abundance of the family Planococcaceae ($P = 0.071$) on D 18. The relative abundance of the families Lachnospiraceae and Ruminococcaceae was quadratically increased due to the TA supplementation. On D 36 but linearly decreased the relative abundance of the families Christensenellaceae and Erysipelotrichaceae ($P < 0.05$). The TA supplementation linearly increased the relative abundance of the family *Bacillaceae* ($P < 0.01$). The TA supplementation linearly ($P < 0.05$) and quadratically (tendency; $P = 0.051$) increased the relative abundance of the family Lachnospiraceae, and the TA2 group had significantly higher relative abundance of the family Lachnospiraceae compared to the TA0.25 and TA0.5 groups.

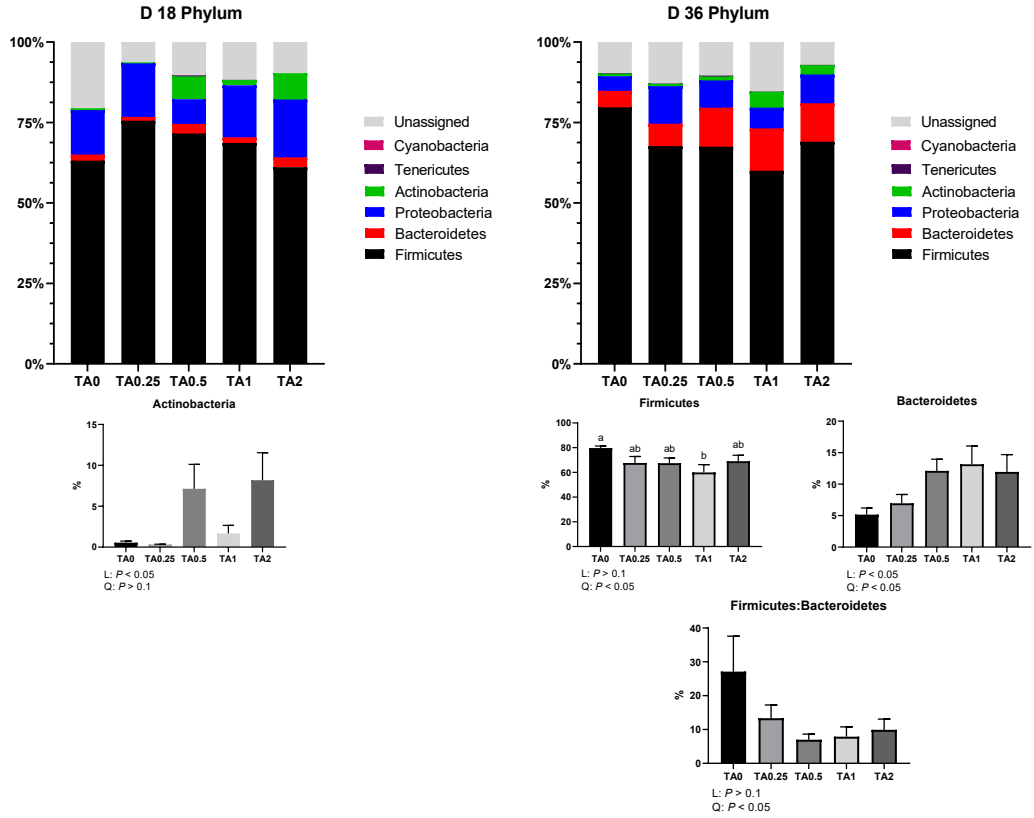

Figure 7.4. Phylum-level composition of the cecal microbial communities in the TA0 (tannic acid 0; basal diet), TA0.25 (tannic acid 0.25; basal diet + 0.25 g/kg TA), TA0.5 (tannic acid 0.5; basal diet + 0.5 g/kg TA), TA1 (tannic acid 1; basal diet + 1 g/kg TA), and TA2 (tannic acid 2; basal diet + 2 g/kg TA) groups on D 18 and 36. All treatment groups were compared using PROC MIXED followed by the Tukey's individual comparison test. Orthogonal polynomial contrasts were used to evaluate the significance of linear or quadratic effects of TA supplementation in broilers.

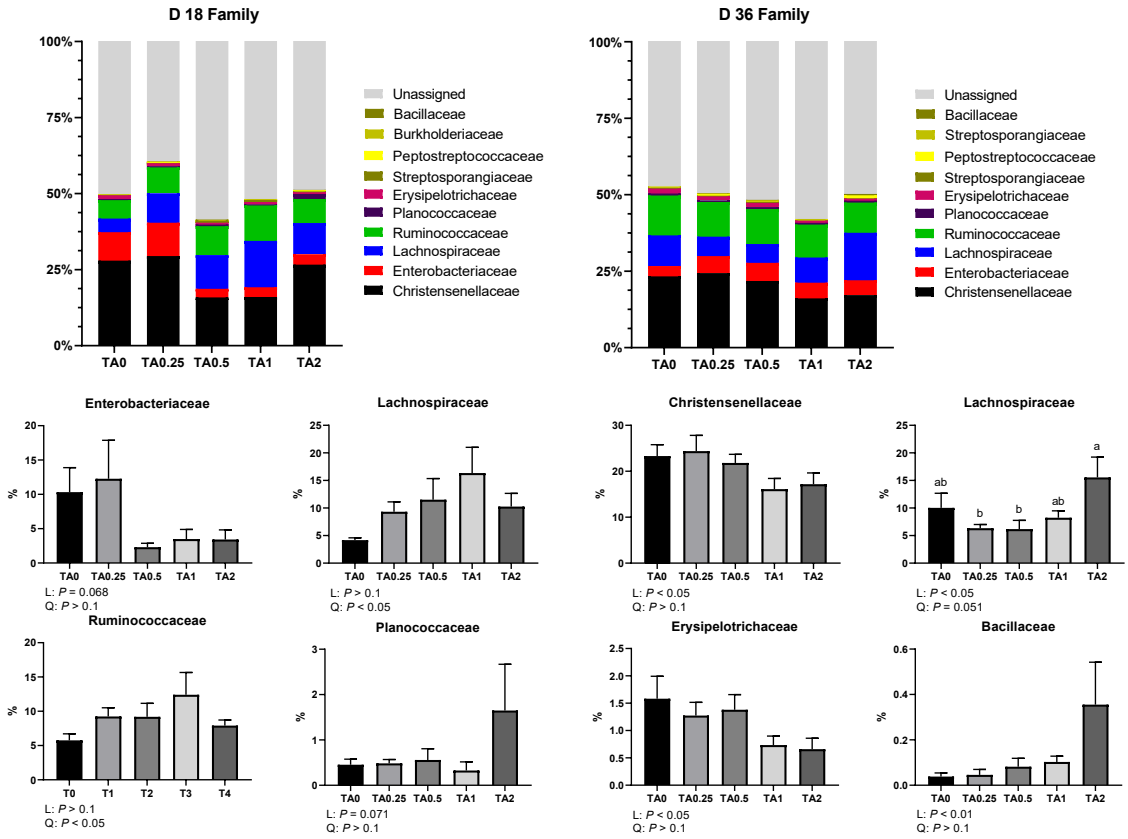

Figure 7.5. Family-level composition of the cecal microbial communities in the TA0 (tannic acid 0; basal diet), TA0.25 (tannic acid 0.25; basal diet + 0.25 g/kg TA), TA0.5 (tannic acid 0.5; basal diet + 0.5 g/kg TA), TA1 (tannic acid 1; basal diet + 1 g/kg TA), and TA2 (tannic acid 2; basal diet + 2 g/kg TA) groups on D 18 and 36. All treatment groups were compared using PROC MIXED followed by the Tukey's individual comparison test. Orthogonal polynomial contrasts were used to evaluate the significance of linear or quadratic effects of TA supplementation in broilers.

7.4.9. Litter ammonia concentration and foot pad dermatitis

There were no differences in litter ammonia concentrations among the treatments on D 42 (Figure 7.6.). The TA1 group had a significantly higher FPD score compared to the TA2 group on D 42. However, no differences were observed in the incidence of FPD among treatments on D 42 ($P > 0.1$).

7.4.10. Bone health parameters and body composition

The TA supplementation linearly reduced BMD ($P < 0.01$) and BMC ($P < 0.05$), and the TA2 group had lower BMD (tendency; $P = 0.051$) and BMC ($P < 0.05$) compared to the TA0 group ($P < 0.05$) on D 42 (Table 7.9.). The body fat percentage was linearly increased by the TA supplementation ($P < 0.05$), and the TA supplementation tended to reduce lean weight ($P = 0.065$). The lean:fat was linearly reduced by the TA supplementation ($P < 0.05$).

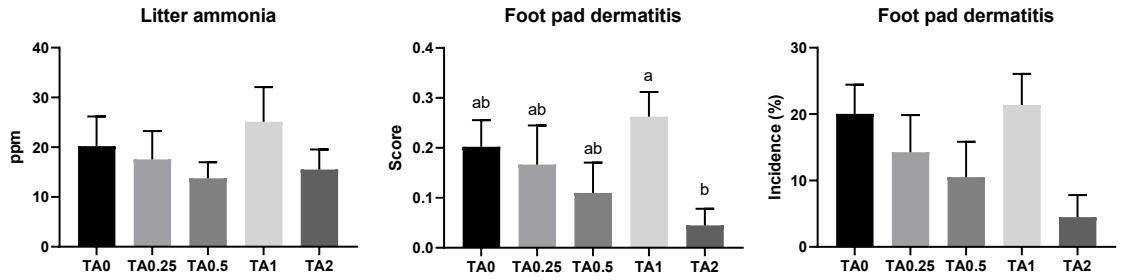

Figure 7.6. Litter ammonia concentration (mg/kg) and foot pad dermatitis score and incidence (%) in the TA0 (tannic acid 0; basal diet), TA0.25 (tannic acid 0.25; basal diet + 0.25 g/kg TA), TA0.5 (tannic acid 0.5; basal diet + 0.5 g/kg TA), TA1 (tannic acid 1; basal diet + 1 g/kg TA), and TA2 (tannic acid 2; basal diet + 2 g/kg TA) groups on D 42. For litter ammonia concentration, all treatment groups were compared using PROC MIXED followed by the Tukey's individual comparison test, and different letters in the same row means significant differences ($P < 0.05$) among treatments. Orthogonal polynomial contrasts were used to evaluate the significance of linear or quadratic effects of supplementation. Foot pad dermatitis score were analyzed using the Kruskal–Wallis test followed by the Dwass–Steel–Critchlow–Fligner post hoc test.

Table 7.9. Bone health parameters including bone mineral content (BMC; g), bone mineral density (BMD; g/cm^2) and body composition parameters including tissue weight (g), lean weight (g), fat weight (g), body fat percentage (%), and lean:fat (g/g) in broilers fed diets supplemented with tannic acid on D 42

								Polynomial contrast[3]	
Items	TA0	TA0.25	TA0.5	TA1	TA2	SEM	*P* value[2]	Linear	Quadratic
BMC	585.71^{a}	546.57^{ab}	508.29^{ab}	525.43^{ab}	464.86^{b}	59.76	0.011	0.001	0.516
BMD	216.14^{a}	204^{ab}	206.43^{ab}	205.71^{ab}	199.71^{b}	9.97	0.051	0.017	0.428
Tissue weight	3668.44	3444.03	3388.12	3462.08	3304.22	359.7	0.430	0.141	0.596
Fat	668.38	644.74	677.21	712.4	686.23	84.33	0.663	0.401	0.435
Fat percentage	18.39	18.81	19.93	20.79	20.73	2.02	0.113	0.022	0.155
Lean weight	2980.71	2799.29	2710.91	2749.55	2616.1	310.87	0.285	0.065	0.465
Lean:Fat	4.5	4.38	4.02	3.9	3.84	0.55	0.115	0.021	0.194

[1]TA0 (tannic acid 0; basal diet); TA0.25 (tannic acid 0.25; basal diet + 0.25 g/kg TA); TA0.5 (tannic acid 0.5; basal diet + 0.5 g/kg TA); TA1 (tannic acid 1; basal diet + 1 g/kg TA); and TA2 (tannic acid 2; basal diet + 2 g/kg TA).

[2]Treatment groups were compared using PROC MIXED followed by the Tukey's individual comparison test. Different letters in the same row means significant differences ($P < 0.05$) among the treatments.

[3]Orthogonal polynomial contrasts were conducted to assess the significance of linear or quadratic effects of TA supplementation in broilers.

7.4.11. Hot weight, abdominal fat, chilled weight, and meat portion

On D 43, hot weight was linearly decreased by the TA supplementation ($P < 0.05$) (Table 7.10.). The TA supplementation linearly increased abdominal fat weight ($P < 0.05$) and abdominal fat percentage ($P < 0.01$). The TA2 group had significantly higher abdominal fat weight compared to the TA1 group had significantly higher fat percentage compared to the TA0.25 and TA1 groups. Total chilled weight was linearly reduced by the TA supplementation ($P < 0.05$), whereas proportion of leg weight was increased by the TA supplementation ($P < 0.05$).

7.4.12. Breast muscle myopathies and meat color (L, a, b), pH, drip loss, thawing loss, and cooking loss in the breast meat

No differences were observed in average breast muscle myopathy scores for white striping, woody breast, spaghetti meat, or hemorrhagic lesions as shown in Figure 7.7. The TA supplementation linearly reduced pH of the breast meat ($P < 0.05$; Table 7.11.) and linearly increased redness (a*) ($P < 0.05$). The TA2 group had significantly greater redness value compared to the TA0 and TA0.5 groups. The TA supplementation linearly (tendency; $P = 0.086$) and quadratically ($P < 0.05$) reduced cooking loss in the breast meat.

Table 7.10. Hot weight, abdominal fat (g and %) weight and meat portion in broilers fed diets supplemented with tannic acid on D 43[1]

								Polynomial contrast[3]	
Items	TA0	TA0.25	TA0.5	TA1	TA2	SEM	*P* value[2]	Linear	Quadratic
Hot weight (g)	2890.9	2873.5	2798.0	2841.7	2772.6	177.61	0.184	0.045	0.710
Abdominal fat (g)	41.9	40.5	42.7	39.5	49.4	11.45	0.072	0.026	0.098
Abdominal fat (%)	1.45	1.41	1.52	1.39	1.78	0.385	0.015	0.005	0.092
Total chilled weight	2926.1	2899.3	2820.71	2866	2793.72	181.45	0.144	0.036	0.621
Legs (%)	26.94	27.36	27.83	27.50	28.43	1.78	0.120	0.017	0.911
Breast (%)	27.10	26.29	26.24	26.16	25.87	2.18	0.483	0.149	0.434
Tender (%)	5.08	4.91	4.72	4.95	4.90	0.65	0.536	0.725	0.383
Wings (%)	9.77	9.80	10.05	9.89	9.96	0.57	0.490	0.378	0.452
Skeleton (%)	31.11	31.64	31.14	31.51	30.83	2.55	0.863	0.575	0.521

[1]TA0 (tannic acid 0; basal diet); TA0.25 (tannic acid 0.25; basal diet + 0.25 g/kg TA); TA0.5 (tannic acid 0.5; basal diet + 0.5 g/kg TA); TA1 (tannic acid 1; basal diet + 1 g/kg TA); and TA2 (tannic acid 2; basal diet + 2 g/kg TA).

[2]Treatment groups were compared using PROC MIXED followed by the Tukey's individual comparison test.

[3]Orthogonal polynomial contrasts were conducted to assess the significance of linear or quadratic effects of TA supplementation in broilers.

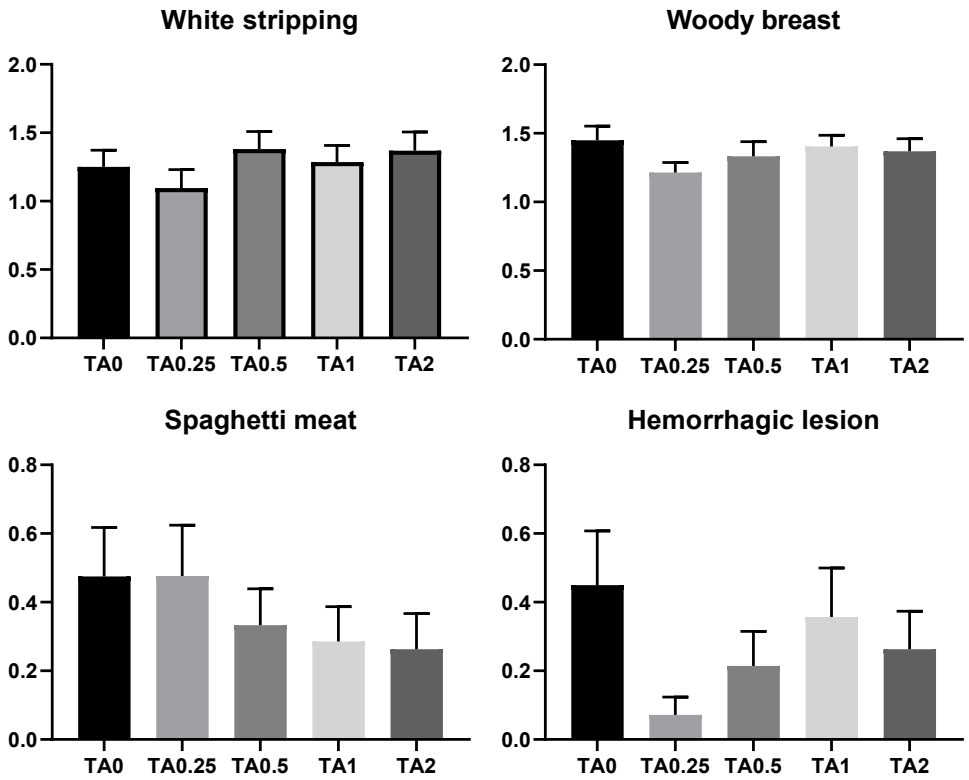

Figure 7.7. Breast muscle myopathies including white striping, woody breast, spaghetti meat, and hemorrhagic lesion in the TA0 (tannic acid 0; basal diet), TA0.25 (tannic acid 0.25; basal diet + 0.25 g/kg TA), TA0.5 (tannic acid 0.5; basal diet + 0.5 g/kg TA), TA1 (tannic acid 1; basal diet + 1 g/kg TA), and TA2 (tannic acid 2; basal diet + 2 g/kg TA) groups on D 42. Score for each parameter was analyzed using the Kruskal–Wallis test followed by the Dwass–Steel–Critchlow–Fligner post hoc test.

Table 7.11. The pH, meat color (L*, a*, b*), drip loss (%), thawing loss (%), and cooking loss (%) in the breast meat of broilers fed diets supplemented with tannic acid[1]

								Polynomial contrast	
Items	TA0	TA0.25	TA0.5	TA1	TA2	SEM	*P* value	Linear	Quadratic
pH	6.02	6.06	6.02	5.99	5.94	0.12	0.152	0.021	0.773
Lightness (L*)	58.21	57.46	57.64	59.24	58.42	2.21	0.206	0.280	0.473
Redness (a*)	0.62^{b}	0.95^{ab}	0.75^{b}	1.04^{ab}	1.75^{a}	0.87	0.015	<0.001	0.535
Yellowness (b*)	13.53	13.72	13.05	14.27	14.35	13.53	0.280	0.105	0.971
Drip loss	3.12	3.10	2.95	2.81	2.36	2.24	0.912	0.330	0.937
Thawing loss	2.60	2.67	2.70	3.00	2.53	1.13	0.873	0.708	0.476
Cooking loss	30.16	30.65	30.89	31.81	28.06	3.45	0.106	0.086	0.027

[1]TA0 (tannic acid 0; basal diet); TA0.25 (tannic acid 0.25; basal diet + 0.25 g/kg TA); TA0.5 (tannic acid 0.5; basal diet + 0.5 g/kg TA); TA1 (tannic acid 1; basal diet + 1 g/kg TA); and TA2 (tannic acid 2; basal diet + 2 g/kg TA).

[2]Treatment groups were compared using PROC MIXED followed by the Tukey's individual comparison test. Different letters in the same row means significant differences ($P < 0.05$) among treatments.

[3]Orthogonal polynomial contrasts were conducted to assess the significance of linear or quadratic effects of TA supplementation in broilers.

7.5. DISCUSSION

Our previous study showed that higher than 1 g/kg TA exhibited antinutritional effects to reduce growth performance, whereas 0.5 g/kg TA increased antioxidant capacity in broilers on D 21 (Choi et al., 2022c). The TA supplementation (0.5 to 2.75 g/kg) improved gut barrier integrity and decreased oocyst shedding in broilers infected with *Eimeria maxima* (Choi et al., 2022b). The TA supplementation (1 to 2 g/kg) enhanced growth performance and gut health via antimicrobial and immunostimulatory effects in broilers infected with *Salmonella* Typhimurium (Choi et al., 2022a). Based on our previous studies, we aimed to evaluate the efficacy of the TA supplementation in broilers raised in floor pens in the current study to simulate actual conditions of broiler production. Therefore, the purpose of the study was to investigate the effects of the TA supplementation (up to 2 g/kg TA) on growth performance, intestinal morphology, activities of brush border digestive enzymes, AID of nutrients, relative mRNA expression of tight junction proteins and nutrient transporters, liver antioxidant capacity, bone health, body composition, and meat yield and quality in broilers on D 42.

In the current study, diets were crumbled and pelleted in the starter and grower/finisher phases, respectively. Almost all commercial broiler feeds are crumbled or pelleted in current broiler production (Brickett et al., 2007). The pelleting processing includes steaming (e.g., conditioning) with high temperature and pelleting (e.g., agglomeration) to produce large particles from small particles (Falk, 1985). In these harsh conditions, stability and molecular or physical traits of feed additives can be altered (Choi et al., 2020b). Kim et al. (2010) reported that thermal process (e.g., autoclave heat) improved antioxidant capacity and antimicrobial effects of TA, and our unpublished data showed that pelleting temperature (80°C) improved antimicrobial effects of TA against *S.* Typhimurium in *in vitro* conditions. However, steaming

and agglomeration during feed processing may induce interactions of TA and nutrients (e.g., proteins, polysaccharides, etc.), which can decrease nutrient utilization in the gastrointestinal tract of chickens.

In the starter and grower phases, BW and feed efficiency were linearly reduced, but no statistical differences were observed in the finisher phase in broilers fed dietary TA in the current study. These results had different trends from our previous studies as follows. Choi et al. (2022c) reported that higher than 1 g/kg TA started to linearly reduce BW of broilers on D 21. However, growth retardation effects of TA were exhibited from 0.25 g/kg TA in the current study. Potentially, the pelleting process may have induced the interaction between TA and dietary nutrients (e.g., proteins). Although AID was not measured on D 18 in the current study, nutrient digestibility would have been severely decreased by the TA supplementation. However, the TA supplementation improved AID of DM, OM, ash, and CP on D 36 potentially as compensation effects and did not alter growth performance in the finisher phase of the current study, indicating that older birds have more tolerance to the TA supplementation. Potentially, mature gastrointestinal tracts (e.g., lower pH and higher pancreatic enzymes) may have hydrolyzed TA-nutrient complex in broilers on D 36 (Adamczyk et al., 2011). In contrast, a previous study by Tonda et al. (2018) reported that the TA supplementation extract improved BWG and feed efficiency in cocci-vaccinated (live vaccine) broilers fed pelleted feed on D 0 to 21. This would be because live vaccines are known to spread coccidiosis in a flock and can decrease growth performance, which can provide challenging conditions to chickens, and this indicates the TA supplementation extract could be effective in challenging conditions (Greif, 2000). However, the basal diets included a coccidiostat (monensin sodium; 500 mg/kg) to exclude the anti-coccidial effects of TA and was conducted in a hygienic laboratory scale

facility, and therefore there would be limited gap to improve growth performance of broilers, which potentially explains reduced or maintained growth performance in broilers supplemented with TA in the current study.

On D 18, the TA supplementation linearly increased jejunal VH:CD and quadratically decreased ileal VH:CD along with reduced CD in the current study. Increased VH:CD indicates augmented nutrient digestion and absorption in chickens (Abd El-Hack et al., 2020). However, if increased VH:CD was accompanied with decreased CD, it cannot be considered as beneficial effects because deeper CD suggests more proliferation and differentiation of stems cells, which would move to the tip of the villus (Liu et al., 2020). Potentially, the TA supplementation might have induced malnutrition by decreasing nutrient utilization via forming complex with nutrients (e.g., proteins), which potentially caused an impairment in the development of intestinal morphology (Shinde et al., 2015).

Activities of sucrase, a brush border digestive enzyme, in the jejunum tissue were quadratically increased by the TA supplementation on D 18 in the current study, which suggests that appropriate dosages of TA can still improve gut development in broilers (Yang et al., 2008). Moreover, relative mRNA expression of MUC2 and nutrients transporters including B0AT1, SGLT1, PepT1, and EAAT3 were linearly and quadratically increased by the TA supplementation. These data indicate that nutrient utilization capacity of the jejunum could be enhanced by the TA supplementation, but limited availability of nutrients due to interactions between TA and nutrients would be the main factor to decrease growth performance of broilers in the starter and grower phases. Otherwise, reduced availability of nutrients for intestinal absorption due to the formation of TA-nutrient complexes in the

luminal side may have increased mRNA expression of nutrients as a resistant reaction to increase nutrient absorption in the gastrointestinal tract (Pinheiro et al., 2013).

Tight junction proteins and MUC2 are closely associated with gut barrier integrity of broilers (Choi et al., 2020c). In the present study, the TA supplementation linearly and quadratically increased relative mRNA expression of genes related to gut barrier integrity including ZO2, CLDN2, JAM2, and MUC2 in the jejunum. According to our previous study, the TA supplementation decreased gut permeability in broilers infected with *E. maxima* (Choi et al., 2022b). A previous study by Yu et al. (2020) reported that the TA supplementation improved gut barrier integrity in weaned piglets. These results suggest that the TA supplementation has potential to increase gut barrier integrity in broilers.

There were no differences in TAC, concentrations of GSH and GSSG, activities of SOD in the liver on D 18 and 36 among the treatments in the present study. Many *in vitro* studies showed that TA, a polyphonic compound, has strong antioxidant capacity (Andrade Jr et al., 2005; Gülçin et al., 2010). However, direct antioxidant effects of TA in chickens were in question. This is because TA should stay inside of the chicken body for a sufficient time by maintaining appropriate forms to exhibit antioxidant capacity (Karakaya, 2004). Deposition of TA in the internal organs (e.g., liver) in broiler chickens should be further investigated. Choi et al. (2022c) showed that the TA supplementation at 0.5 g/kg indirectly improved antioxidant system by enhancing activities of SOD in the liver. The differences would be originated from the pelleting process, which may reduce bioavailability of TA by forming TA-nutrient complexes. However, under the heat stress condition, the TA supplementation (10 g/kg) showed potential to improve antioxidant capacity in broilers (Ebrahim et al., 2015).

In the present study, the TA supplementation linearly decreased alpha diversity indices including faith's phylogenetic diversity (communities' evolutionary distance; D 18 and 36), observed features (richness; D 18 and 36), pielou evenness (evenness; D 36), and shannon entropy (richness and evenness; D 36). While it is still controversial, lower alpha diversity may indicate less stable and unmature microbial communities in the gastrointestinal tract of animals (Ebrahim et al., 2015). Moreover, beta diversity indices (unweighted and weighted unifrac) showed that different dosages of TA could modulate cecal microbial communities in broilers.

The relative abundance of the phylum Actinobacteria was linearly increased by the TA supplementation on D 18 in the current study. The phylum Actinobacteria includes Bifidobacteria species, which can improve gut barrier integrity and immune system of animals and is considered as a beneficial phylum in animals (Binda et al., 2018). On D 18, the TA supplementation linearly reduced relative abundance of the family Enterobacteriaceae, which includes diverse pathogens such as *Salmonella* spp., *Shigella*, *Escherichia coli*, etc. Consistently, our previous study reported that the TA supplementation reduced cecal *Salmonella* Typhimurium load in the starter phase of broilers (Choi et al., 2022a). Moreover, the TA supplementation quadratically increased the relative abundance of the families Lachnospiraceae and Ruminococcaceae, which have an important role in maintain gut homeostasis by producing volatile fatty acid via fiber degradation (Biddle et al., 2013). Consistently, Koo and Nyachoti (2019) reported that the TA supplementation enhanced cecal volatile fatty acid production in pigs. However, on D 36, a ratio of the phyla Firmicutes and Bacteroidetes was quadratically reduced by the TA supplementation in the current study. The lower ratio of the phyla Firmicutes and Bacteroidetes suggests a lower capacity of fiber degradation and production of short chain fatty acids, important energy sources for the host

animals (Singh et al., 2012). In the current study, the TA supplementation linearly decreased the relative abundance of the families Christesenellaceae and Erysipelotrichaceae, which have an important role in fiber degradation to produce short chain fatty acids (Wasti et al., 2021). However, the TA2 group significantly increased the relative abundance of the family Lachnospiraceae compared to the TA0.25 and TA0.5 groups, and the TA supplementation linearly increased the relative abundance of the family Bacillaceae, which are positively correlated with growth performance and feed efficiency (Moula et al., 2018). While the TA supplementation reduced the relative abundance of the families Christensenellaceae and Erysipelotrichaceae, the TA supplementation still increased the relative abundance of the families Lachnospiraceae and Bacillaceae in the current study.

Ammonia (NH3) in poultry houses can negatively affect the health of chickens and humans as well as harm the environment (Naseem and King, 2018). Chickens synthesize uric acid as the end product of purine and protein metabolism, and uric acid is converted into ammonia via microbial fermentation in the ceca or in the litter (Kim and Patterson, 2003a; Kim and Patterson, 2003b; Naseem and King, 2018). In the current study, we hypothesized that litter ammonia concentration could be reduced by the TA supplementation because the TA supplementation increased AID of CP on D 36. Crude protein digestibility is closely associated with litter ammonia concentration (Brink et al., 2022). Moreover, Arzola-Alavarez et al. (2020) reported that the addition of pine bark tannin in the litter reduced ammonia accumulation in the poultry litter. Unabsorbed TA could be excreted to the litter and potentially modulate ammonia concentration in the litter. However, no differences were observed in the litter ammonia on D 42. The TA1 group had a significantly higher FPD score compared to the TA2 group, which implies that the TA supplementation can modulate FPD score in broilers. Litter

ammonia and FPD are closely associated (Youssef et al., 2011), and our current study also showed that litter ammonia and FPD score had similar trends. Otherwise, BW could simply affect litter ammonia because bigger birds would excrete more manure in the litter. The TA1 group had numerically close BW compared to the TA0 group, whereas the TA2 group had the numerically lowest BW on D 42. These results may explain the trends of litter ammonia and FPD score of broilers fed diets supplemented with TA in the current study.

Our previous study showed that there was only a linear tendency ($0.05 < P \leq 0.10$) to reduce bone health parameters including BMD and BMC in broilers on D 21, but no statistical differences were observed between the groups fed 2.5 g/kg TA and 0 g/kg TA (Choi et al., 2022c). However, in the present study, BMD and BMC were linearly reduced by the TA supplementation in broilers, and 2 g/kg TA supplementation significantly reduced BMD and BMC compared to the control group on D 42. Possibly, the TA supplementation might have reduced utilization of calcium, phosphorous, and iron, which are important minerals for bone formation in broilers (Afsana et al., 2004; Hassan et al., 2003; Katsumata et al., 2009; Shang et al., 2015b). Moreover, the pelleting process may have induced more formation of TA-mineral complexes, which dramatically reduced BMD and BMC in broilers. A previous study by Tomaszewska et al. (2018) also reported that inclusion of low-tannin faba bean (condensed tannins) negatively affected tibia traits (weight, reduction of the cross section area, and wall thickness) in broilers.

In the present study, the fat percentage measured by DEXA was linearly increased and the ratio of lean to fat decreased in broilers on D 42. This result is in stark contrast to our previous results reporting that the TA supplementation increased the ratio of lean to fat in broilers on D 21 (Choi et al., 2022c). Discrepancies between the findings of these studies could

have originated from differences in the supplementation period and age of birds (D 42 v. D 21) and feed form (pelleted v. mash). In our previous study (Choi et al., 2022c), fat accumulation was reduced in broilers D 21 due to less production of cecal volatile fatty acids, which potentially resulted in an imbalance of energy homeostasis. However, in the current study, the pelleting process may have decreased nutrient utilization by inducing the formation of TA-nutrient complexes, and young broilers, which did not have mature enough gastrointestinal tract to hydrolyze TA-nutrient complexes, may have undergone malnutrition (e.g., energy deficiency). Energy deficiency could have increased feed intake in broilers to meet their energy requirement (Choi et al., 2021). However, because TA can decrease feed intake of broilers by inducing astringent taste in broilers (Choi et al., 2022b), energy deficiency caused by the TA supplementation did not lead to an increase of feed intake in broilers. Instead, after broilers experienced malnutrition, the broilers may have altered their body metabolism to increase the accumulation of fat to cope with energy deficiency by the TA supplementation. A number of studies showed that malnutrition can induce an increase of fat accumulation in the body (Kobylińska et al., 2022). Consistently, a previous study by Starčević et al. (2015) also showed that the TA supplementation (5 g/kg) increased fat accumulation in the breast and thigh meat in broilers on D 35.

In the current study, absolute and relative weight of abdominal fat were increased by the TA supplementation, which is consistent with DEXA results. The abdominal fat weight is a dependable parameter to represent body fat content because abdominal fat is the main and largest area (up to 4 % of BW) of fat accumulation in broilers (Fouad and El-Senousey, 2014; Thomas et al., 1983). Furthermore, the TA supplementation resulted in linearly increased leg meat and linearly decreased breast meat yield (P = 0.149), while statistical differences were

not observed. Leg meat had higher fat content compared to breast meat (Pikul et al., 1985). Potentially, increased fat metabolism in broiler body by the TA supplementation resulted in increased leg meat yield and decreased breast meat yield to increase fat accumulation in chickens. Fatty broiler meat and low yield of breast meat are not preferred in modern broiler production (Fouad and El-Senousey, 2014). Low pH of the breast meat results in higher lightness, lower redness, and higher yellowness by decreasing water binding capacity (Allen et al., 1997; Qiao et al., 2001). Our current study showed that the TA supplementation decreased pH, increased redness, and reduced cooking loss in the breast meat. This would be due to immatureness of breast meat and low growth performance caused by the TA supplementation. According to Bianchi et al. (2007), smaller broilers had lower pH and higher redness when compared to the bigger broilers. However, the lightness, an important factor to indicate pale, soft, and exudative (PSE)-like condition in poultry meat (Petracci et al., 2004), was not modulated due to the TA supplementation in the current study. Moreover, the TA supplementation did not dramatically altered those meat quality parameters, and the values were in still normal range (Hertanto et al., 2018). No differences were observed in the breast muscle myopathies such as white striping, woody breast, spaghetti meat, and hemorrhagic lesion in the current study. Therefore, the TA supplementation did not significantly modulate meat quality of broiler chickens.

In the current study, contradictory results from our previous studies and negative effects of the TA supplementation would be mainly attributed to the pelleting process on diets, which induced the formation of TA-nutrient complexes. In order to minimize or inhibit the interaction of TA and dietary nutrients during pelleting process, the encapsulation of TA can be a potential strategy (Choi et al., 2020b). Encapsulation process can provide protection for

TA and release TA in the target site of the gastrointestinal tract, where many pathogens propagate (e.g., lower gut) (Choi et al., 2020b). A previous study by Wang et al. (2020b) reported that encapsulated TA showed beneficial effects on gut health and microbiota of weaned piglets. Future studies should include: 1) investigation of appropriate methods to encapsulate TA and its stability during pelleting process and in the gastrointestinal tract; and 2) investigation into the effects of encapsulated TA on growth performance and gut health in broilers on D 42.

7.6. CONCLUSION

The TA supplementation up to 2 g/kg in pelleted diets positively affected gut microbiota, enhanced brush border digestive enzyme activities, upregulated genes related to gut barrier integrity and nutrient transportation in the starter/grower phases, and improved nutrient digestibility in the finisher phase. However, the TA supplementation decreased overall growth performance and feed efficiency, increased fat accumulation, and negatively affected gut microbiota, bone health, and meat production in broilers on D 42. Therefore, further processing should be applied on TA to enhance their potential beneficial effects on broilers.

8.0. CHAPTER 8 GENERAL DISCUSSION AND CONCLUSION

8.1. GENERAL DISCUSSION

Our ultimate goal in the current project was to evaluate tannic acid (TA) as an alternative for antibiotic growth promotors (AGP) for the purpose of developing an antibiotic-free broiler production system. The efficacy of TA was assessed in *in vitro* and *in vivo* conditions including general and bacterial and parasitic infection conditions. Initially, the potential antimicrobial effects of TA at lethal and sublethal concentrations in *in vitro* conditions, and intestinal delivery of TA in the gastrointestinal tract of broiler chickens were evaluated. This was decided as the antimicrobial effect of TA would be the one of the main mode of actions to exhibit growth and gut health promoting effects in broiler chickens (Redondo et al., 2014a). TA exhibited antimicrobial effects against *S.* Typhimurium and other *Salmonella enterica* serotypes, and MIC against ST^{NR} was determined as 40 mg/kg. Moreover, approximately 10% of TA was able to localize to the ceca, the GI site for many pathogens including *S.* Typhimurium in broilers. By using the MIC and recovery rate of bioactive compounds in the gastrointestinal tract, the optimal concentration of a bioactive compound capable to inhibit antimicrobial effects can be approximated (Yang et al., 2016b). In our study, 400 mg/kg TA was the minimum concentration capable of displaying antimicrobial activity against *S.* Typhimurium in broiler chickens.

We next surveyed different concentrations of TA on growth performance, gut health, and gut microbiota in broiler chickens on D 21. The reasons included 1) TA can show beneficial or negative effects depending upon concentration in animals (e.g., hormesis); and 2) results in an *in vitro* gastrointestinal tract can be very different from an *in vivo* study with live chickens. In Chapter 2, we found that TA at >1 g/kg TA can yield anti-nutritional effects non-

challenged D21 broilers. Whereas TA supplementation at 500 to 1,000 mg/kg enhanced activities of superoxide dismutase in the liver and positively affected gut microbiota, it failed to improve growth performance and other gut health parameters in broiler chickens.

When the birds were challenged with *S.* Typhimurium, TA supplementation improved growth performance and gut health, potentially by reducing *S.* Typhimurium colonization in the ceca and stimulating host immunity. However, TA's antimicrobial effects were not observed until concentrations exceeded 1 g/kg TA. This would suggests that the estimated concentration based on the MIC against *S.* Typhimurium and intestinal delivery rate of TA can be different from the optimal concentration determined in *in vivo* broilers whereas the values were only two fold different (400 mg/kg v. 1,000 mg/kg). This would be mainly because the MIC was determined *in vitro*, and there are many other factors (other pathogens, interaction with host cells, etc.) in *in vivo* gastrointestinal that can affect antimicrobial effects of TA (Kowalska-Krochmal and Dudek-Wicher, 2021).

In the current project, TA supplementation exhibited anti-parasitic effects against *E. maxima* by decreasing oocyst shedding of *E. maxima* and attenuating damaged gut permeability in broilers infected with *E. maxima*. *E. maxima* was specifically selected for the study because 1) *E. maxima* colonize the jejunum, which is the main area for nutrient digestion and absorption (Teng et al., 2021a); and 2) enough amount (around 54%) was recovered in the jejunum part of broilers. Since TA supplementation had beneficial effects in both a bacterial and parasitic challenge model, future studies are needed to explore the efficacy of TA supplementation under a co-infection model [e.g., bacterial (*Salmonella* spp. and *Campylobacter jejuni*) and *Eimeria* infection model and necrotic enteritis model] in broiler chickens.

TA supplementation at 500 to 1,000 mg/kg increased antioxidant capacity and favorably modulated gut microbiota in broilers under healthy condition, which suggests that broilers fed diets supplemented with TA may be more resistant or resilient against challenging conditions. Still, significant growth promoting effects were not observed in broilers on D 21 and 42 under the non-challenge condition. Thus, TA supplementation should be accompanied with appropriate diagnostic systems to maximize broiler production efficiency (Fatoba and Adeleke, 2018). This is because, not all broilers in the field are raised in the challenging conditions (Johar et al., 2021; Liu et al., 2019), and TA supplementation does not display growth promoting effects under these conditions and may even have anti-nutritional effects in chickens. Hence, appropriate TA concentrations in the feed should be adjusted based on the specific conditions in the broiler production to maximize production efficiency.

Compromised bone health associated with dietary TA would be one of the obstacles to TA supplementation in broiler production. Our studies showed that TA supplementation linearly reduced bone mineral density in healthy broilers on D 21 and 42 under normal operating conditions. Compromised bone health may be associated with mineral (e.g., iron, phosphorous, etc) precipitation capacity of TA, which would be related to antimicrobial effects of TA against *Salmonella* spp. (Johar et al., 2021). Iron is essential for Salmonella growth and plays important role in bone formation in animals (Abbas et al., 2013). Thus, the stronger antimicrobial effects associated with TA supplementation would be likely accompanied by compromised bone health in broilers. However, 1 g/kg TA in feed showed a significant reduction ($p < 0.05$) in ST^{NR} load in the ceca and in oocyst shedding of *E. maxima* without a significant reduction in bone mineral density of broilers.

Another potential challenge to routine TA supplementation in broiler production would be interaction of TA with nutrients during the pelleting process. TA forms complexes with dietary nutrients (e.g., proteins, minerals, and polysaccharides) during the pelleting process, which agglomerates small particles into large particles with steam and heat. This may explain the reported impaired growth performance, bone health, and meat quantity and quality in TA feed supplemented healthy broilers on D 42. Moreover, these results contrast with our previous studies (chapter 5 and 6) which reported that ~1 g/kg TAin the feed showed growth and gut health promoting effects in broilers on D 21. Because most commercial poultry feed is pelleted (Zaefarian et al., 2021), novel dietary formulations are needed in order to utilize TA and to maximize their beneficial effects in broiler production by minimizing interactions of TA with dietary nutrients during pelleting process. Suggested pre-treatments of TA would include 1) encapsulation of TA (Wang et al., 2020b); and 2) combining TA with other nutrients (e.g., bovine serum albumin) (Lee et al., 2010). These pre-treatments would protect TA from combining with other nutrients and release TA in the target site in the gastrointestinal tract. Appropriate encapsulation methods and optimal concentrations of protected TA should be determined to apply TA in broiler production.

8.2. GENERAL CONCLUSION

TA supplementation at ~1 g/kg in feed displayed anti-nutritional effects in healthy broilers. Further, in broilers infected with *S.* Typhimurium, TA supplementation at 2 g/kg showed antimicrobial and growth promoting effects. In addition, TA supplementation at 2.75 g/kg exhibited anti-parasitic effects and attenuated damaged gut barrier integrity without affecting growth performance of broilers infected with *E. maxima*. However, healthy D42 broilers, which were fed pelleted feed supplemented with TA in the floor pen, growth

performance was reduced, and body composition and meat quality and quantity of broilers were negatively modulated. Hence, while TA supplementation can be beneficial to attenuate reduced growth performance and compromised gut health in broilers when challenged with bacterial and parasitic challenging conditions, their efficacy can be limited in healthy birds. Therefore, more studies are required to maximize their efficacy under field conditions.

9.0. CHAPTER 9 FUTURE DIRECTIONS

Based on our current project, future directions include:

1. To further investigate the effects of TA supplementation on growth performance, oocyst shedding, gut health, gut microbiota, bone health, and body composition in D 42 broilers infected with *Eimeria* spp. or under necrotic enteritis;
2. To further study the effects of TA supplementation on growth performance, gut health, Salmonella colonization in the gastrointestinal tract and meat in D 42 broilers infected with *Salmonella* spp.;
3. To find appropriate encapsulation methods for TA, and to investigate its stability during pelleting process and in the gastrointestinal tract of broilers.;
4. To determine optimal concentrations of encapsulated TA in broilers under healthy and challenged conditions (e.g., bacterial and parasitic infection, heat stress, etc.).

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