Evaluating specific B vitamins concentration on growth performance, digestibility and intestinal integrity in poultry

by

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B.S., University of Missouri, 2019
M.S., Iowa State University, 2021

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Grain Science and Industry
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Abstract

Optimizing nutrient utilization and gastrointestinal health is essential to maximizing growth performance and production efficiency in modern poultry systems. Feed costs account for up to 70% of the modern production costs and therefore nutritional strategies that improve ingredient utilization, and digestive function can enhance nutrient efficiency to maximize growth performance and bird health. Modern poultry production requires increasingly precise nutritional and feed manufacturing strategies to maximize growth performance, nutrient utilization, and gastrointestinal health while maintaining economic efficiency. Improvements in genetic potential of broilers and turkeys have increased metabolic demands, placing greater emphasis on vitamin adequacy, ingredient utilization, and digestive efficiency to support optimal performance. In particular, the role of vitamin nutrition in energy metabolism, the use of alternative feed ingredients, and the interaction between nutrient digestibility and gastrointestinal health remain key areas of investigation. Therefore, the objectives of this dissertation were to evaluate the role of specific vitamin B concentrations in broiler and turkey nutrition (Chapters 1, 2, and 3), and assess sorghum particle size effects on broiler growth performance and digestive physiology (Chapter 4).

To achieve these objectives, multiple experiments were conducted in broilers and turkeys. The first study evaluated the effects of niacin (NA) and the emerging NA analog nicotinamide riboside (NR) on growth performance, energy digestibility, and intestinal morphology in broilers (Chapter 1, Exp 1). Supplementation of NA above a basal level of 28 mg/kg or NR addition, did not affect body weight gain (BWG), feed intake (FI), or feed conversion ratio (FCR; $P > 0.05$), and apparent metabolizable energy (AME) was not improved with additional NA ($P > 0.05$). In contrast, NR supplementation linearly decreased apparent total

tract digestibility of gross energy (P = 0.02) and AME (P = 0.02). While NA increased villus height (quadratically, P = 0.04) and NR increased crypt depth (quadratically, P = 0.01). A second broiler study evaluated increasing NA inclusion from 28 to 268 mg/kg (Chapter 1, Exp 2) and observed a quadratic response in AME (P = 0.08), with no differences in AME up to 208 mg/kg and then reduced at 268 mg/kg (P = 0.02), while growth performance remained unchanged (P > 0.05). These findings suggest that within the limitations of these battery studies, current NA recommendations are adequate for broiler growth and that NR can substitute NA in the diet without negatively impacting growth performance. The third study evaluated NA and NR supplementation in turkey hens from 0 to 42 days of age to determine whether increased NA inclusion improves growth performance and intestinal morphology (Chapter 2). Turkeys fed an additional 70 mg/kg NA demonstrated improved FCR (P = 0.02), while additional 70 mg/kg NR showed a tendency for improved FCR (P = 0.07), with no differences in BWG or FI (P > 0.05). Total dietary NA levels reached 172 mg/kg, exceeding current requirement estimates and suggesting that modern turkey hens may benefit from higher NA inclusion to support feed efficiency.

The fourth study investigated riboflavin supplementation in turkey starter diets during summer conditions to evaluate its impact on growth performance and intestinal permeability (Chapter 3). Increasing dietary riboflavin from 11.24 to 28.10 mg/kg resulted in no differences in BWG, FI, or FCR for d 1-21 (P > 0.05). From d 22-43 and overall, there were no linear or quadratic responded in BWG or FI, however, FCR tended to quadratically respond (P = 0.064). There was no response detected in fluorescein isothiocyanate–dextran intestinal permeability with increasing riboflavin inclusion (P > 0.05) even after a period of environmental conditions of >32℃ and >50% relative humidity for 5 days. These results suggest that dietary riboflavin

inclusion of 11.24 mg/kg is sufficient during the summer months with no negative impact on growth performance or intestinal permeability.

The fifth study evaluated sorghum particle size effects on broiler growth performance and digestive physiology from 4 to 49 days of age (Chapter 4). Broilers fed sorghum-based diets had greater BWG and FI compared to those fed corn-based diets ($P < 0.05$), with no differences in FCR ($P > 0.05$). Increasing sorghum particle size resulted in a tendency for increased FI (linear, $P = 0.059$) and BWG (quadratic, $P = 0.056$), with optimal performance observed between 1,000 and 1,200 µm. Coarser particle size supported improved gizzard development and growth performance, demonstrating that feed manufacturing practices play a critical role in optimizing alternative grain utilization.

Overall, the results presented here with in this dissertation demonstrate that optimizing vitamin inclusion and feed processing provides practical and mechanistic framework to improve poultry performance and intestinal health. These findings support evidence-based vitamin recommendations for modern broilers and turkeys, validate sorghum as a viable alternative energy source when properly processed, and emphasize the importance of precision nutrition to enhance metabolic efficiency and intestinal health. Integrating metabolic nutrition with feed manufacturing practices provides poultry nutritionists and feed manufacturers with actionable strategies to improve feed efficiency, reduce production costs, and enhance modern poultry production.

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Major Professor
Dr. Chad Paulk

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Chapter 1 - Niacin or nicotinamide riboside supplementation on growth performance, apparent metabolizable energy, and intestinal morphology in broilers

ABSTRACT

Adequate niacin (NA) levels in broiler diets play a crucial role in enhancing growth performance and efficient utilization of digestible energy and minerals. Nicotinamide riboside (NR), an NA analog, has emerged as an alternative NA feed additive. Two experiments were conducted to investigate the effects of supplementing varying NA and NR levels or increasing NA levels on growth performance, intestinal morphology and energy digestibility in broilers. Both studies utilized day-old Cobb by-product breeders (420 broilers) in either an 18-d (Exp 1) or 21-d (Exp 2) study where five dietary treatments were randomly assigned to 60 cages (7 birds/cage) within 12 location blocks across 3 batteries. Each set of experimental treatments were a sublot of a basal diet mix which contained 28 mg/kg NA. Dietary treatments in Exp. 1consisted of either basal diet, or the addition of NA or NR at 42 and 112 mg/kg diet, where dietary treatments in Exp 2 consisted of a titration of 28, 88, 148, 208, or 268 mg NA/kg diet. In Exp. 1, there was no difference in overall body weight gain (**BWG**), feed intake (**FI**) or feed efficiency (**FCR**) between treatment level or niacin source ($P > 0.05$). The addition of NA had no impact on AME ($P > 0.05$) however the addition of NR linearly decreased ATTD of GE ($P = 0.02$) and AME ($P = 0.02$). The addition of NA quadratically increased villus height ($P = 0.04$), and the addition of NR quadratically increased crypt depth ($P = 0.01$) while villus to crypt ratio was not impacted by NA or NR at either level ($P > 0.05$). In the Exp. 2, there were no differences in BWG, FI or FCR across all dietary treatments ($P > 0.05$).

However, AME tended to respond quadratically ($P = 0.08$) up to 208 mg/kg NA, decreasing at 268 mg/kg NA ($P = 0.02$). Within the limitations of these studies, the recommended NA supplementation of 28 mg/kg appears to be sufficient to optimize growth performance and energy digestibility of the diet.

INTRODUCTION

Niacin (NA, vitamin B_3) is a water-soluble vitamin and is known for its crucial role of contributing to the production of nicotinamide adenine dinucleotide (**NAD+**) which is a required cofactor in several energy metabolic processes (e.g., Krebs cycle, electron transport chain, and glycolysis). Through these pathways, feeding the appropriate level of NA maintains growth performance, metabolism, and overall health through the maintenance of oxidation and reduction reactions (Bogan and Brenner, 2008). Although NA is largely present in corn, only 30% is biologically available due to being bound by hemicellulose and proteins (Yen et al., 1977). Using this availability value and given that corn contains approximately 24 mg NA per kg (NRC, 2012), this results in approximately 7 mg of NA being available per kg of corn. Because recommendations of NA for starter and grower broilers range from 30 to 84 mg/kg NA (NRC, 1994; Aviagen, 2022; Cobb-Vantress, 2022; DSM-Firmenich, 2022), there is a need further to further define the need for NA supplementation in the diet associated with its contribution to the NAD+ pool.

Niacin is not the only pathway in which NAD+ can be synthesized, with 3 pathways known to contribute to the NAD+ pool in the body. The first is via NA, through the Preiss-Handler pathway. Secondly, tryptophan (**Trp**) can *de novo* synthesize NA through the Kynurenine pathway, which downstream contributes to NAD+ but this pathway contributes to less than 1% of the NAD+ pool. Thirdly, nicotinamide riboside (**NR**) is a structural analog to

NA, which can contribute to the NAD+ pool through the Salvage pathway and may be more efficient than NA contributing through the Preiss-Handler pathway (Bogan and Brenner, 2008).

A purified form of NR has recently emerged as an alternative NA feed additive, having been suggested to act similarly to NA as both are precursors to NAD+ (Yang et al., 2007; Zhang et al., 2016). Previous literature reports *in ovo* feeding of NR to broilers lead to increased muscle fiber production and increased body weight gain at 28, 42 and 47 days compared to birds that did not receive NR (Xu et al., 2021; Maynard et al., 2024). However, NR has not been used in the diet of newly hatched broilers.

A recent vitamin survey (DSM-Firmenich, 2022) concluded that producers were feeding 42-52 mg/kg NA which is above the NRC (1994) recommendations but below genetic and vitamin supplier recommendations (Cobb-Vantress, 2022). Presently, vitamin and specifically NA recommendations vary across literature and genetic suppliers, while analogs like NR may be able to replace NA supplementation. With industry wide advancements in genetics, analog synthesis, and minimally published vitamin nutrition research, there is lack of peer reviewed recommendations and understanding of NA and NR relationships in broiler feed formulations. Therefore, the objective of these studies was to determine the effect of added NA or NR and NA level on growth performance, intestinal morphology and apparent metabolizable energy (AME) in broiler chicks.

MATERIALS AND METHODS

Two experiments were conducted at Kansas State University Poultry Unit in Manhattan, KS, using experimental protocols and methods approved by the Institutional Animal Care and Use Committee (4850-29520).

Experiment 1

Experiment 1 (Exp 1) was conducted to determine the effects of the addition 42 or 112 mg/kg of NA or NR in the diet and its impact on growth performance, AME and intestinal morphology in broiler chicks. Dietary treatments consisted of a basal corn and soybean meal-based diet (28 mg/kg NA) and the control plus 42 mg/kg or 112 mg/kg of NA (ROVIMIX ® Niacin, 99.5%, DSM-Firmenich, Parsippany, NJ, USA) and 42 mg/kg or 112 mg/kg NR h(NIAGEN®, ChromaDex Ingredients, Los Angeles, CA, USA). Research feed was manufactured at O. H. Kruse Feed Mill, in Manhattan, KS. Diets were formulated using the recommendations set forth by Cobb-Vantress (2022) for all nutrients besides vitamins and minerals (Table 1). The basal control diet was mixed in a single batch utilizing a 907 kg twin shaft counterpoise mixer (Hayes and Stoltz, Model TRDB63-0152, Fort Worth, TX). A vitamin and mineral premix (NB3000, NutraBlend, Neosho, MO) was included at 2.27 kg per ton to provide 28 mg/kg of NA. The basal mix was divided into 5 lots, 136 kg each, to be used for each treatment. Treatments were manufactured by adding either NA or NR to the basal lots and mixing (Model S3, H.C. Davis Sons Mfg. Co. Inc., Bonner Springs, KS) for an additional 7 min with feed recycled through the mixer three times, evenly spaced during the mixing period to prevent dead space build up. Diets were fed in mash form.

Exp 1 utilized a total of 420 one-day-old male Cobb by-product breeder (Cobb-Vantress, Siloam Springs, AR) in an 18-d study. Chicks were housed in 3 batteries, with the 5 dietary treatments randomly assigned to 60 cages within 12 locational blocks, resulting in 12 replicates per treatment and 7 chicks per cage at placement. Battery cages were 96.5 × 33 cm, and chicks were maintained in an environmentally control room with *ad libitum* access to feed and water and a lighting schedule of 22L:2D for the duration of the study. Pen weight and feed consumption were measured on d 10 and 18 for calculation of BWG, FI and FCR. In the case of mortality or removal, chick weight, treatment, cage number, and date of mortality were recorded. Reported FI and FCR were adjusted for mortality and bird days.

After weighing on d 18, two chicks per pen were sacrificed via CO_2 asphyxiation and distal sections of the ileum were collected for morphology assessment. Tissue samples were taken in 3 cm sections, approximately 3 cm proximal to the ileo-cecal junction. Upon collection, ileum samples were rinsed with distilled water and submerged in 10% w/v formalin solution. The tissues were stained with hematoxylin and eosin (Iowa State University Veterinary Diagnostic Laboratory, Ames, IA). The resulting slides were analyzed for crypt depth (CD) and villus height (VH; OLYMPUS BX 53/43 microscope with an attached DP80 Olympus camera, Waltham, MA), where 10 well-defined villus and crypt pairs with proper orientation were measured per chick (OLYMPUS cellSens Dimension 1.16 software), averaged, and reported as 1 value per chick. Villus were selected equally around the entire cross-section, and for each VH measured, the CD to the right was also measured. The average VH and CD per chick was also used to calculate a ratio (V:H).

Energy digestibility was measured using the total collection methodology where total excreta samples were collected from lined battery pans once daily on d 16, 17, and 18 of the study. These samples were weighed and frozen at -20°C until drying. Samples were dried at 55°C over 72 hours. Dried samples were ground, and a representative sample was analyzed for gross energy (**GE**). Gross energy of the feed and excreta was determined using an isoperibol bomb calorimeter (Model 1281, Parr Instrument Co., Moline, IL) using benzoic acid as a standard, in duplicate. The total tract coefficient of digestible energy obtained was used to determine energy digestibility, where AME_{total} was calculated by using FI (kg), E is excreta output (kg), GE_{feed} is the gross energy (kcal/kg) of feed, $GE_{excreta}$ is the gross energy (kcal/kg) of excreta (eq. 1).

$$\mathrm{AME}_{total} = \left(\frac{FI \times GE_{(feed)} - E \times GE_{(excreta)}}{kg\ FI}\right) \qquad \text{(eq. 1)}$$

Experiment 2

Experiment 2 (Exp 2), treatment additions of NA (ROVIMIX ® Niacin, 99.5%, DSM-Firmenich, Parsippany, NJ, USA) were made through hand addition being mixed with a portion of the basal diet into the total dietary treatment. Treatments were in 60 mg/kg increments, resulting in NA supplementation of 28, 88, 148, 208, and 286 mg/kg in the complete diet. Titanium dioxide was included in all diets at 0.5% as an indigestible marker. Feed samples were collected at the time of production. The basal diet was mixed in a two 567 kg batches utilizing a 907 kg twin shaft counterpoise mixer (Hayes and Stoltz, Model TRDB63-0152, Fort Worth, TX). The basal mix was divided into 5 lots of 227 kg of basal diet for each treatment allocation. Due to all treatments being from the same batch of basal feed, the assumption is made that there are equal levels of available NA in the diet composition before supplementation of treatments. Dietary treatments were made by mixing each 227 kg lot with the respective treatment. Mash diets were steam conditioned (25 × 140 cm Wenger twin shaft pre-conditioner, Model 150, Sabetha, KS) and pelleted using a 1-ton 30-horsepower pellet mill (1012-2 HD Master Model, California Pellet Mill, Crawfords, IN) equipped with a 4.4 × 35 mm (length: diameter 8) pellet die. Prior to pelleting, the conditioner was set for a conditioning retention time of 30 sec. Target conditioning temperature range was 82-85°C. Temperature readings were recorded three times per batch for conditioning temperature and hot pellet temperature. Pellets were cooled using a 1-ton capacity counter flow cooler (OP><FLO™ AIR COOLER, Bliss Industries, Ponca City, OK) for 15 min prior to sampling and bagging. Diets were fed in crumble form utilizing a 23 × 15 cm single pair 3-horsepower roller mill (A24013, RMS, Harrisburg, SD).

A total of 420 one-day-old male Cobb by-product breeder (Cobb-Vantress, Siloam Springs, AR) were used in a 21-d study. Chicks were housed in 3 batteries, with the 5 dietary treatments that were randomly assigned to 60 cages within 12 locational blocks, resulting in 12 replicates per treatment and 7

chicks per cage at placement. Chicks were maintained in the same room conditions and lighting schedule as Exp 1. Pen weight and feed consumption were measured at d 21 for calculation of BWG, FI and FCR. In the case of mortality or removal, chick weight, treatment, cage number, and date of mortality were recorded. Reported FI and FCR were adjusted for mortality and bird days.

For Exp 2, grab samples of total excreta were collected from battery pans once daily for d 19, 20 and 21 of the study. These samples were weighed and frozen at -20℃ until drying. Samples were dried at 55℃ over 72 hours. Dried samples were ground, and a representative sample was analyzed for titanium dioxide and GE. Gross energy of feed and excreta were analyzed with the same methodology as described previously. Titanium dioxide was analyzed based on previously outlined methods (Leone, 1973), where feed and excreta samples were ashed in an oven and then digested with sulfuric acid and hydrogen peroxide, followed by measuring absorbance using a UV spectrophotometer against a standard curve. AME was calculated using GE_{feed} as the GE (kcal/kg) of feed $GE_{excreta}$ as the GE (kcal/kg) of excreta, $TiO_{2\ feed}$ as titanium dioxide of feed and $TiO_{2excreta}$ is titanium dioxide of excreta (eq. 2).

$$\mathrm{AME}_{index} = \mathrm{GE}_{feed} - \left(\mathrm{GE}_{\ (\mathrm{excreta})} \times \frac{\mathrm{TiO}_{2(\mathrm{feed})}}{\mathrm{TiO}_{2(\mathrm{excreta})}}\right) \qquad \text{(eq. 2)}$$

Statistical analysis

Data were analyzed as a randomized complete block design using the PROC GLIMMIX of SAS 9.4 (SAS Institute Inc., Cary, NC, USA) with cage as the experimental unit and pen location as the blocking factor. For Exp. 1, orthogonal polynomial contrasts were used to assess the effects of (1) NA vs NR, (2) linear and quadratic effects of increasing NA, and (3) linear and quadratic effects of increasing NR. For Exp. 2, orthogonal polynomial contrasts were used to assess the linear and quadratic effects of increasing NA. Results were considered significant if $P \leq 0.05$ and tendencies if $0.05 < P \leq 0.10$.

RESULTS AND DISCUSSION

For Exp. 1, there was no evidence of linear or quadratic response in BWG, FI, and FCR for broilers fed diets containing NA compared to those fed diets containing NR ($P > 0.05$; Table 2). Similarly, there was no evidence of linear or quadratic response in BWG and FCR for broilers fed containing increasing concentrations of NA or NR. However, broilers fed diets containing 42 mg/kg NR had decreased FI compared to those fed the control or 112 mg/kg NR diets (quadratic, $P = 0.05$). In Exp. 2, there was no evidence of differences ($P = 0.43$) in BWG, FI and FCR in broilers fed diets with increasing concentrations of NA in the diet from 28 to 268 mg/kg (Table 3). Niacin feeding recommendations for broilers range greatly across industry as the NRC (1994) recommends 35 mg/kg NA whereas genetic suppliers recommend 50-70 mg/kg (Aviagen, 2022; Cobb-Vantress, 2022). Recently, an industry wide vitamin survey was completed and determined that on average, broiler nutritionists are formulating starter and grower diets to contain 47-52 mg/kg NA (DSM-Firmenich, 2022). In the present studies, the control diet contained 28 mg/kg NA added via the vitamin trace mineral premix with approximately 13-14 mg/kg available NA coming from dietary ingredients.

Niacin: Previous research evaluating NA supplementation in broilers have reported variable responses. An earlier study concluded increased BWG when feeding broilers 66 mg/kg and 99 mg/kg NA compared to 33 mg/kg NA, while 99 mg/kg NA improved FCR compared to 33 and 66 mg/kg NA in a 49 day floor pen study (Waldroup et al., 1985). However a later study concluded that broilers should be supplemented 33 mg/kg NA to maximize growth performance and minimize leg disorders from d 1-21 in batteries (Ruiz et al., 1990). Oloyo (2000) observed increased BWG and FI in broiler chicks supplemented 37.5 mg/kg NA compared to 15, 22.5 and 30 mg/kg NA, with no further improvement at 45, 52.5 and 60 mg/kg NA in a 42-d floor pen study. Most recently, when feeding fast growing broilers 60 mg/kg NA improved ADG, ADFI, and GF in a 56 d study (Jiang et al., 2011).

Collectively, these studies indicate that responses to supplemental NA vary across experiments with differences among studies likely reflecting variation in broiler genetics, dietary composition, housing systems, and experimental duration. Earlier work was frequently conducted with slower-growing genotypes or under different nutritional conditions, whereas modern broilers exhibit greater growth rates and metabolic demands. In the present study, feeding more than 28 mg/kg NA did not result in significant differences in broiler growth performance. Numerical increases in FCR could be of economic importance; however, these studies are limited to an 18 and 21 d study using battery cages. Nicotinamide Riboside: Previous research investigating the supplementation of NR in poultry is limited. The present study is the first known to utilize NR in a broiler feeding study. However, NR has been studied in pigs and broiler embryos. *In ovo* feeding of NR to developing broiler embryos increased pectoralis major muscle development compared with controls (Xu et al. 2021). These responses were associated with increased expression of genes involved in muscle growth and cell cycle regulation, suggesting that NR enhances NAD-dependent metabolic pathways regulating myogenesis. Short-term NR supplementation in finishing pigs prior to harvest improved mitochondrial integrity and slowed apoptotic activation in muscle tissue, which contributed to improved pork color stability and postmortem muscle metabolism (Zhu et al., 2025). Collectively, these findings indicate that NR supplementation can influence muscle metabolism and mitochondrial function in livestock species by increasing NAD+ availability and enhancing oxidative metabolic pathways which could lead to improved growth performance.

In Exp 1, feeding 42 and 112 mg/kg NR did not result in significant differences in broiler growth performance, but feeding 112 mg/kg NR did result in a 4% numerical improvement in BWG and FCR in broilers fed mash diets. Numerical increases in FCR again could be of economic importance, however, Exp 1 is limited to an 18 d study using battery cages which is different than commercially grown boilers using wood shavings commercial barn, longer feeding periods, and different

feed form and water systems. That said, if dietary NA supply is already sufficient to support NAD+ synthesis, further supplementation of NR may not provide additional metabolic benefits.

Energy digestibility

Apparent metabolizable energy is the measure of the disappearance of energy that is consumed from the feed. If there is an increase in absorbative capacity, there may be an increase in AME due to increased disappearance of energy. For Exp. 1, the addition 42 and 112 mg/kg NA did not impact dietary AME ($P > 0.05$, Table 4). However, the addition of NR from 42 to 112 mg/kg linearly decreased dietary AME ($P = 0.02$) and ATTD of GE ($P = 0.02$). In Exp 2, dietary AME was similar up 208 mg NA/kg diet supplementation and decreased when NA reached 268 mg/kg ($P = 0.02$; Table 5) indicating NA supplementation tended to quadratically decrease ATTD of GE ($P = 0.08$). For ATTD of GE, there was a negative linear relationship with increasing levels of NA ($P < 0.001$).

The current study evaluated NA and NR based on their physical weights, were the addition of 42 or 112 mg/kg diet. However, their molecular weight differs with NA having a molecular weight of 123.11 g/mol and NR-Cl having a molecular weight of 290.7 g/mol, resulting in NA having 2.3 more molecules per gram in physical weight than NR-Cl. Consequently, when NA is digested, it can passively diffuse across the enterocyte as NA whereas NR requires transporter-mediated uptake and enzymatic metabolism to enter the enterocyte (Sadoogh-Abasian and Evered, 1980). Therefore, the linear decrease in AME with the additional NR from 42 to 112 mg/kg could be explained by the need for breakdown with fewer molecules delivered to the NAD+ pool resulting in a lower net ATP production.

Niacin functions as a precursor for NAD+, which is a required cofactor for numerous metabolic reactions involved in carbohydrate, lipid, and protein metabolism. Under conditions where NA supply is limiting, additional supplementation could theoretically enhance metabolic efficiency and improve nutrient utilization. However, when NA intake greatly exceeds physiological requirements, excessive NAD-precursor availability could disrupt metabolic regulation or alter nutrient partitioning. For example,

Adebowale et al. (2019) reported increased AME in turkey toms when dietary NA was increased from 26 to 86 and 206 mg/kg NA supplementation Ivers and Veum (2012) observed no change in ME when increasing NA concentrations in growing pigs at 6.0, 10.0, 14.0, 18.0, 22.0, or 44.0 mg/kg NA for a 35-d experiment These contrasting responses suggest that the effects of NA on energy utilization may depend on species, diet composition, and baseline nutrient supply. The reduction in energy digestibility in Exp 1 with the 112 mg/kg NR treatment and Exp 2 at the 268 mg/kg NA may indicate that excessive NAD precursors alter intestinal metabolic activity, nutrient transport processes, metabolic feedback mechanisms that regulate NAD synthesis and utilization when precursor supply is excessive (Augspurger and Baker, 2007). Moreover, limitations of the present studies may be that diets were formulated to meet the energy requirements of the broilers and therefore there was no improvements to growth or energy digestibility to be gained from the additional NAD-precursor in the diet.

Intestinal morphology

Intestinal morphology can be related to the capability and activity of digestion and absorption in the gastrointestinal tract. The measure of VH is indicative of the intestinal absorbative capacity while CD is indicative of intestinal cellular turnover. An increase in VH suggests increased surface area and absorbative capacity of nutrients in the intestine while a decrease in VH suggests a decrease in capacity or intestinal damage. An increase in CD suggests cellular damage in the intestine as the crypt is responsible for the production and differentiation of enterocytes. The crypt cells directly feed into the cells that make up the villi. Therefore, we hypothesized that with increasing NAD+ available to the energy producing processes in the cell, VH would increase with no effect on CD.

In Exp 1, intestinal morphology and observed VH responded quadratically to added NA where VH increased at 42 mg/kg added NA ($P = 0.03$) with no further increase at 112 mg/kg NA

supplementation. However, CD did not respond to added NA but responded quadratically to added NR where CD increased up to NR1 ($P = 0.01$) When observing V:C, there were no responses to added NA or NR ($P > 0.05$). Niacin or NR may influence intestinal morphology, as it serves as a precursor for the coenzymes NAD+, which is essential for cellular energy metabolism and redox reactions involved in enterocyte proliferation and differentiation (Yi et al., 2021). The intestinal epithelium has a high rate of cell turnover, with epithelial cells proliferating in the crypts and migrating along the villi as they mature. Adequate NAD-precursor supplementation supports ATP production and metabolic activity required for epithelial turnover, which may promote increased VH and reduce excessive CD, thereby improving the V:C and absorptive capacity. Consequently, increasing dietary NAD-precursor has the potential to support intestinal epithelial development and nutrient absorption, particularly when NA supply is limiting or when intestinal tissues are under metabolic or inflammatory stress. Yi et al. (2021) observed various changings in small intestinal morphology with increasing levels from 22-75 mg/kg NA in weaned pigs while Liu et al. (2021) observed no impact of 20 mg/kg NA supplementation compared to no supplementation on ileal VH, but a decrease in CD with no overall V:C difference in weaned pigs. Overall, Exp 1 is in contrast with previous studies in pigs suggesting dietary supplementation of NA might not modulate broiler intestinal cell proliferation, as it does in pigs. However, 112 mg/kg NR supplementation decreased both VH and CD compared to 42 and 0 mg/kg NR supplementation. This indicates NR may have interrupted cellular proliferation differently than NA. Additionally, the short duration of the present experiments and the lack of nutritional or environmental stressors may have limited the potential for detecting changes in intestinal structure.

It is also important to consider with NA and NR supplementation with respect to the Trp level in the diet because Trp can contribute to NAD+ pool. Tryptophan can serve as a metabolic precursor for NA through the Kynurenine pathway thereby influencing NAD+ precursor requirements. When dietary Trp is limiting, a greater proportion of Trp may be diverted toward NA synthesis rather than protein accretion, potentially increasing the dietary requirement for NA (Baker et al., 1973). Thus, when adequate Trp is supplied in the diet, endogenous NA synthesis may partially contribute to meeting metabolic needs. Previous studies have demonstrated that deficiencies in either Trp or NA can impair growth performance in poultry due to their interconnected roles in NAD metabolism (Czarnecki et al., 1983; Ruiz and Harms, 1990). In the present experiments, diets were formulated to meet Trp requirements for broilers at 0.24% Trp, which may also contribute to the lack of response to supplementation of NA or NR.

In conclusion, niacin is a required vitamin in the diet due to its essential function contributing to the production of NAD+ but it is not yet understood how it may impact the metabolism of energy and subsequent growth responses when supplemented over the minimum requirement in the diet of broilers. Within the limitations of the studies reported herein, the recommended NA supplementation of 28 mg/kg appears to be sufficient to maximize BWG, FI, FCR, and dietary AME. It is worthy to note that NR is not a heat stable feed additive and therefore what benefits in FCR it may provide as demonstrated in the current studies, may not be practical in application for broilers which typically consume pelleted diets. Future research with NR may focus on layers as they are fed mash diets. However, further research is needed to observe NA supplementation in a full grow out period of broilers in a commercial setting. It would be of added interest to evaluate the economic return of the numerical FCR improvements on the additional NA supplementation.

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Table 1.1 Experimental basal diet formulations.[1]

Ingredient	Experiment 1	Experiment 2
Corn, 650 µm	54.17	59.35
Soybean meal, 46% CP	35.83	35.16
Corn DDGS[2], 3-6%	5.00	-
Limestone	0.90	0.87
Dicalcium phosphate	1.81	1.88
NaCl	0.40	0.40
L-Lysine•HCl	0.22	0.14
DL-Methionine	0.29	0.28
L-Threonine	0.06	0.10
Choline chloride, 60%	0.06	0.06
Phytase[3]	0.008	0.008
Soybean oil	1.00	1.00
Vitamin mineral premix[4]	0.25	0.25
Total	100.00	100.00
Calculated composition		
Crude protein	24.08	22.87
Tryptophan	0.24	0.24
Niacin, mg/kg	28.00	28.00
AME, kcal/kg	2,922	3,035

[1] Diets were formulated to meet the genetic supplier nutrient recommendations, excluding vitamins due to the nature of the study. One basal diet was mixed and subdivided into 5 lots for treatment additions.
[2] Dried distillers grains with solubles
[3] Quantum Blue 10G (AB Vista, Plantation, FL) 10,000 FTU/g of additive.
[4] NB3000 (NutraBlend, Nevada, MO) provided 40 g zinc, 20 g iron, 40 g manganese, 4500 ppm copper, 600 ppm iodine, 60 ppm selenium, 3,087,000 IU vitamin A, 1,102,500 IU vitamin D_3, 6,615 IU vitamin E, 331 mg vitamin K, 441 mg vitamin B_1, 2,646 mg vitamin B_2, 11,023 mg vitamin B_3, 2,646 mg vitamin B_5, 551 mg vitamin B_6, 13 mg vitamin B_7, 276 mg vitamin B_9, 4.41 mg vitamin B_{12}, and 154,323 mg choline per kg of premix.

Table 1.2 Exp 1 Effect of added NA or NR on growth performance in broiler chicks by phase[1]

								NA		NR	
Item	Control[2]	NA1[3]	NA2[3]	NR1[4]	NR2[4]	SEM	NA vs NR	Linear	Quadratic	Linear	Quadratic
d 0-18											
BWG, g	721	724	728	708	749	19.7	0.88	0.79	0.99	0.24	0.31
FI, g	927	931	910	891	927	15.2	0.42	0.36	0.57	0.76	0.05
FCR, g/g	1.29	1.29	1.25	1.26	1.24	0.026	0.43	0.19	0.69	0.14	0.73

[1] Male broilers (n = 420, Cobb byproduct broiler, Cobb-Vantress, Siloam Springs, AR) were placed in battery cages at hatch with 7 broilers per cage and 12 replicates per treatment. Performance criterion was measured from d 0 to 18.
[2] The VTM provided 28 mg niacin/kg diet.
[3] The VTM provided 28 mg niacin/kg diet with 42 or 112 mg niacin (NA)/kg of diet added to provide a total of 70 or 140 mg niacin/kg of diet (ROVIMIX® Niacin, 99.5%, DSM-Firmenich, Parsippany, NJ, USA).
[4] The VTM provided 28 mg niacin/kg diet with 42 or 112 mg nicotinamide riboside (NR)/kg diet added to provide a total of 28 mg niacin/kg diet and 42 or 112 mg NR/kg diet (NIAGEN®, ChromaDex Ingredients, Los Angeles, CA).
[5] Linear and quadratic interactions were tested.
[abc] Superscript indicates treatment differences $P < 0.05$.

Table 1.3 Exp 2 Effect of niacin level on growth performance in broiler chicks d 0-21.

	Niacin concentration in the diet, mg/kg[2]						Statistics		
Item	28	88	148	208	268	SEM	Linear	Quadratic	*P*-value
BWG, g	939	958	962	947	959	21	0.65	0.69	0.93
FI, g	1,017	1,038	1,019	1,018	1,060	29	0.43	0.55	0.75
FCR, g/g	1.13	1.11	1.11	1.11	1.11	0.01	0.49	0.47	0.78

[1] 420, day old, male broilers (Cobb by-product broiler, Cobb-Vantress, Siloam Springs, AR) were placed in 60 battery cages (N = 7) resulting 12 replicates per treatment. Growth performance was measured for day 0-21.

[2] The VTM provided 28 mg/kg niacin, additional niacin was added to achieve treatment concentrations (ROVIMIX® Niacin, 99.5%, DSM-Firmenich, Parsippany, NJ, USA).[3] Linear and quadratic contrasts were evaluated for increasing NA in the diet

Table 1.4 Exp 1. Apparent metabolizable energy of diets containing added NA or NR in broilers[1]

Item	Control[2]	NA1[3]	NA2[3]	NR1[4]	NR2[4]	SEM	*Lin*, NA	*Quad*, NA	*Lin*, NR	*Quad*, NR	*P*-value
Feed intake, g	1,579	1,632	1,491	1,579	1,594	81.9	0.37	0.40	0.89	0.96	0.81
GE intake, kcal/kg	6,213	6,410	5,801	6,213	6,284	321.5	0.30	0.38	0.86	0.94	0.73
GE output, kcal/kg	1,654	1,738	1,529	1,638	1,731	90.5	0.25	0.24	0.51	0.69	0.48
ATTD of GE, %	73.4	72.9	73.7	73.6	72.3	0.43	0.49	0.26	0.02	0.20	0.08
AME_{total}, kcal/kg	3,290	3,254	3,266	3,296	3,238	19.4	0.42	0.21	0.02	0.25	0.12

[1] Male broilers (n = 420, Cobb byproduct broiler, Cobb-Vantress, Siloam Springs, AR) were placed in battery cages at hatch with 7 broilers per cage and 12 replicates per treatment. Performance criterion was measured from d 0 to 18.
[2] The VTM provided 28 mg niacin/kg diet.
[3] The VTM provided 28 mg niacin/kg diet with 42 or 112 mg niacin (NA)/kg of diet added to provide a total of 70 or 140 mg niacin/kg of diet (ROVIMIX® Niacin, 99.5%, DSM-Firmenich, Parsippany, NJ, USA).
[4] The VTM provided 28 mg niacin/kg diet with 42 or 112 mg nicotinamide riboside (NR)/kg diet added to provide a total of 28 mg niacin/kg diet and 42 or 112 mg NR/kg diet (NIAGEN®, ChromaDex Ingredients, Los Angeles, CA).
[5] Linear and quadratic interactions were tested.
[abc] Superscript indicates treatment differences $P < 0.05$.

Table 1.5 Exp 2. Effect of niacin level on apparent metabolizable in broiler chicks[1].

	Niacin concentration in the diet, mg/kg[2]								
Item	28	88	148	208	268	SEM	*Lin.*	*Quad.*	*P*-value
AME_{index}, kcal/kg	3,454	3,403[ab]	3,461[a]	3,469[a]	3,356[b]	26.7	0.12	0.08	0.02
ATTD of GE, %	78.0[a]	76.0[b]	76.1[b]	76.7[ab]	73.9[c]	0.59	<0.01	0.59	< 0.01

[1]420, day old, male broilers (Cobb by-product broiler, Cobb-Vantress, Siloam Springs, AR) were placed in 60 battery cages (*N* = 7) resulting 12 replicates per treatment. Growth performance was measured for d 0-21. Excreta was collected on d 17 and 18, analyzed for titanium dioxide and gross energy, and AME was calculated utilizing the index methodology.
[2] The VTM provided 28mg/kg niacin and 60 mg/kg of niacin (ROVIMIX® Niacin, 99.5%, DSM-Firmenich, Parsippany, NJ, USA) was added to additional treatments to achieve titration.
[3] Linear and quadratic contrasts were evaluated for increasing NA in the diet
[a-c] Difference in superscript within row indicates $P \leq 0.05$

Table 1.6 Exp 1 Effect of added niacin or nicotinamide riboside on ileal histology in broiler chicks[1].

								NA		NR	
Item, µm	Control[2]	NA1[3]	NA2[3]	NR1[4]	NR2[4]	SEM	NA vs NR	Linear	Quadratic	Linear	Quadratic
Villus height	574.5[ab]	623.8[a]	552.2[b]	587.3[ab]	537.4[b]	24.33	0.27	0.33	0.04	0.19	0.36
Crypt depth	151.4[ab]	157.1[ab]	152.3[ab]	165.5[a]	143.0[b]	5.52	0.93	0.99	0.39	0.12	0.01
V:C	3.8	3.9	3.7	3.6	3.8	0.1	0.46	0.38	0.21	0.83	0.26

[1] Male broilers (n = 420, Cobb byproduct broiler, Cobb-Vantress, Siloam Springs, AR) were placed in battery cages at hatch with 7 broilers per cage and 12 replicates per treatment. Performance criterion was measured from d 0 to 18.
[2] The VTM provided 28 mg niacin/kg diet.
[3] The VTM provided 28 mg niacin/kg diet with 42 or 112 mg niacin (NA)/kg of diet added to provide a total of 70 or 140 mg niacin/kg of diet (ROVIMIX® Niacin, 99.5%, DSM-Firmenich, Parsippany, NJ, USA).
[4] The VTM provided 28 mg niacin/kg diet with 42 or 112 mg nicotinamide riboside (NR)/kg diet added to provide a total of 28 mg niacin/kg diet and 42 or 112 mg NR/kg diet (NIAGEN®, ChromaDex Ingredients, Los Angeles, CA).
[5] Linear and quadratic interactions were tested.
[abc] Superscript indicates treatment differences $P < 0.05$.

Chapter 2 - Impact of niacin and nicotinamide riboside supplementation on growth performance and intestinal morphology of turkey hens.

ABSTRACT

Niacin (**NA**) is a water-soluble vitamin supplemented to turkey diets being essential for optimal growth performance and health. Nicotinamide riboside (**NR**), an analog to NA, is a new feed additive which contributes to NAD+ similar to NA and may serve a similar function in turkey nutrition as NA. The objective of this study was to determine the effects of added NA or NR to turkey hen diets on growth performance and intestinal morphology. Day-old poults (*N* = 900) were allocated to 30 pens which were randomly assigned one of three dietary treatments consisting of a control diet (102 mg NA/kg diet, 0 mg NR/kg diet), or the control diet with either 70 mg added NA or NR per kg diet. Test articles (i.e., NA or NR) were mixed with 2% soybean oil and added via post pellet liquid application. Diets were fed for 42 d for assessment of BEW, FI, and FCR. For d 0-18 and d 19-42 there was no evidence of differences in BWG, FI or FCR (*P* > 0.05?) due to dietary treatment. Overall, (d 0-42) there was no evidence of differences in BWG or FI between turkeys fed any dietary treatment (*P* > 0.05). Overall, turkeys fed added NA had improved (*P* = 0.02) FCR compared to turkeys fed the control diet, and turkeys fed added NR had a tendency for improved (*P* < 0.07) FCR compared to turkeys fed the control diet. There was no evidence of differences in intestinal morphology measured at the distal ileum (*P* > 0.05). The NRC (1994) suggests the requirement for turkey poults from 0 to 56 d of age is 60 mg

niacin/kg diet; however, this research demonstrates an improvement in FCR when feeding up to 172 mg/kg of NA.

INTRODUCTION

Niacin (**NA**, Vitamin B_3) is a water-soluble vitamin and is known for its crucial role of contributing to the production of nicotinamide adenine dinucleotide **(NAD+;** Kohlmeier, 2003) which is a required cofactor in several energy metabolic processes. Through these pathways, feeding the appropriate level of NA maintains growth performance, metabolism, and overall health through oxidation and reduction reactions (Bogan and Brenner, 2008).

Although NA is present in corn, there is only about 7 mg NA/kg corn available to poultry (Yen et al., 1977). Presently, there are established recommendations for pre-starter and starter turkeys that range 60-160 mg NA/kg diet across the NRC (1994), genetic, and vitamin suppliers (REFS). Therefore, there may be a need for further NA supplementation in the diet to contribute to the NAD+ pool. Niacin is not the only pathway in which NAD+ can be synthesized with 3 pathways contributing to the NAD+ pool in the body. The first is via NA, through the Preiss-Handler pathway, the second utilizes tryptophan (**Trp**) via *de novo* synthesis and third, nicotinamide riboside (**NR**) contributes to the NAD+ pool through the Salvage pathway (Bogan and Brenner, 2008). Tryptophan is presently supplemented in the diet for protein accretion and is less efficient than NA with less than 1% of the NAD+ pool coming from this pathway. In contrast, NR is a structural analog to NA and may be more efficient than NA contributing through the Preiss-Handler pathway (Yang et al., 2007; Zhang et al., 2016). Although NR is not a common feed additive in livestock diets, previous literature reports *in ovo* feeding of NR to broilers lead to increased muscle fiber production and increased body weight gain at 28, 42 and

47 d in compared to birds that did not receive NR (Xu et al., 2021; Maynard et al., 2024). However, NR has not been fed *in ovo* or to hatched turkey poults.

Presently, vitamin and specifically NA recommendations vary across literature and genetic suppliers with the NRC (1994) recommending 60 mg/kg from 1-8 weeks of age. Currently, analogs like NR are becoming available which may be able to replace NA supplementation. With industry wide advancements in genetics and minimal published vitamin nutrition research, there is lack of peer-reviewed recommendations and understanding of NA and NR relationship in modern turkeys. The objectives of this study were to determine the effect of supplemented NA or NR on growth performance and intestinal morphology in commercial turkey hens during the pre-starter and starter phases.

MATERIALS AND METHODS

This experiment was conducted at Kansas State University Poultry Unit in Manhattan, KS, using experimental protocols and methods approved by the Institutional Animal Care and Use Committee (4850-9000).

One-day-old commercial turkey hens (Hybrid Hendrix Genetics, Beatrice, NE) were placed into 30 pens (N = 900) balanced by initial BW resulting in 30 poults per pen. Dietary treatments were randomly assigned to pens within location block, resulting in 10 pens per treatment. Treatments consisted of a basal diet (102 mg/kg NA, 0 mg/kg NR; **CON**), or the CON with 70 mg/kg NA (**+NA**) or NR (**+NR**) added via post pellet liquid application (**PPLA**). The control diet was formulated (Table 1) to meet the recommended nutrient requirements provided by the genetic supplier except for vitamins and minerals which were provided through a commercially available vitamin and trace mineral premix (**VTM**; Table 2). The VTM was included in the diet and met or exceeded the NRC (1994) recommendations for vitamins and

minerals except biotin, folic acid, thiamine and pyridoxine A basal diet was mixed using a 907 kg twin shaft counterpoise mixer (Hayes and Stoltz, Model TRDB63-0152, Fort Worth, TX) and divided into three batches with NA and NR being added via PPLA. All dietary treatments had 2% of the formulated soybean oil added via PPLA, with or without the NA or NR addition. Pellets were crumbled and then weighed out and received PPLA in a mixer for 120 seconds. Pre-starter mash diets were steam conditioned (25 × 140 cm Wenger twin shaft pre-conditioner, Model 150, Sabetha, KS) and pelleted on a 1-ton 30-horsepower pellet mill (1012-2 HD Master Model, California Pellet Mill, Crawfordsville, IN) equipped with a 4.4 × 35 mm (length: diameter 8) pellet die. While starter mash diets were steam conditioned and pelleted using a 100-HP pellet mill (Model 3016-4 Master, California Pellet Mill Co., Crawfordsville, IN), equipped with a 4 × 35 mm die (length: diameter 8.75). Prior to pelleting, conditioners were set for a conditioning retention time of 30 sec. Target conditioning temperatures were 82-85℃ with temperature readings recorded three times per batch for conditioning temperature and hot pellet temperature quality control. Pellets were cooled using a counter flow cooler (OP><FLO™ AIR COOLER, Bliss Industries, Ponca City, OK) for 15 min prior to sampling and bagging. Pre-starter and starter diets were crumbled utilizing a 23 × 15 cm single pair 3-horsepower roller mill (A24013, RMS, Harrisburg, SD).

Turkey poults were housed in floor pens (1.5 × 4.3 m) with unused wood shavings, and each pen was equipped with a single feeder (Chore Time, Milford, IN) and a 4-cup drinker system (Lubing EasyLine, Campodarsego, Italy) allowing for *ad libitum* access to feed and water. Feeder trays were placed for the first three days to facilitate feed intake. Feeders were shaken twice daily to ensure adequate feed flow and waters were cleared of shavings as needed. The environmental temperature was maintained to follow the genetic supplier recommendations

and stair-stepped down from 34℃ to 23℃ over the course of the study. The lighting schedule was stair stepped to 18 h light, and 6 h dark from d 0 to 7 and maintained at 18L:6D at 90 LUX until wk 3 when half of the light bulbs were taken out to reduce lighting to 45 LUX.

Growth Performance

Pen and feeder weights were measured on d 18 and 42 for calculation of BWG, FI and FCR. Initial BW was measured at placement of poults on d 1 to balance locational blocks. In the case of mortality or removal, poult weight, treatment, pen number, and date of mortality were recorded. Reported FI and FCR were adjusted for mortality and bird-days.

Intestinal morphology

Upon the completion of the feeding period on d 42, two birds per pen were euthanized via CO_2 asphyxiation and distal sections of the ileum were collected for determination of morphology. Tissue samples were taken in 3 cm chunks, approximately 3 cm proximal to the ileo-cecal junction. Upon collection, ileum samples were rinsed with distilled water and submerged in 10% w/v formalin solution. Tissues were stained with hematoxylin and eosin (Iowa State University Veterinary Diagnostic Laboratory, Ames, IA). The resulting slides were analyzed for crypt depth and villus height (CD and VH, respectively; OLYMPUS BX 53/43 microscope with an attached DP80 Olympus camera, Waltham, MA). Ten well-defined villus and crypt pairs with proper orientation were measured per chick were averaged and reported as one value per bird. Villus were selected equally around the entire cross-section, and for each VH measured, the CD to the right was also measured. The average VH and CD per chick was also used to calculate a VH:CD ratio.

Statistical Analysis

Data were analyzed as a randomized complete block design using the PROC GLIMMIX in SAS 9.4 (SAS Institute, LLC., Cary, NC) with pen as the experimental unit and pen location in the barn as the blocking factor. Results were considered significant if $P \leq 0.05$.

RESULTS AND DISCUSSION

The present experiment determined that feeding 172 mg NA/kg diet improved FCR in turkeys compared to turkeys fed 102 mg NA/kg diet. From d 0 to 18 and 19 to 42 there was no evidence of differences in BWG, FI or FCR ($P = 0.133$). From d 0 to 42 there was no evidence of differences in BWG or FI ($P = 0.381$) between dietary treatments; however, turkeys fed added NA had improved ($P = 0.02$) FCR compared to those fed the control diet and turkeys fed added NR had a tendency for improved ($P = 0.07$) FCR compared to those fed the basal diet. On note is the NRC (1994) recommends 60 mg NA/kg in the complete diet, and genetic suppliers recommend 70-85 mg NA/kg for commercially fed turkeys from 0–6-wk of age.

Previous research determined that feeding an additional 180 mg NA/kg diet resulted in the highest BWG and best FCR when feeding toms from 4-8 weeks of age without impacting FI, compared to birds fed the control (26.7 mg NA/kg diet) or the addition of 60 mg NA/kg diet (REF). Similar results were observed with the same NA treatments in d 56-112 turkeys where BWG and FI were greatest and FCR was the best when toms were supplemented 180 mg NA/kg diet compared to toms supplemented 0 or 60 mg NA/kg diet (Adebowale et al., 2019a). In another study, turkey toms were supplemented 0, 60 or 180 mg NA/kg diet from d 28-56 where birds fed 180 mg NA/kg diet had the highest BWG, lowest FI, and lowest FCR (Adebowale et al., 2019b). Adebowale et al. (2019b) also observed a positive response in estimation of the dietary apparent metabolizable energy (**AME**) in response to increasing NA, which could be why

the toms had better growth performance. The previous literature and the present study indicate that the NRC (1994) requirements for NA are outdated for the modern genetics currently fed in the United States.

Previous research investigating the supplementation of NR in poultry is limited. The present study is the first known to utilize NR in turkeys. In contrast, NR has been studied in pigs (Hennesy et al., 2024) and in broilers where *in ovo* feeding of NR to developing broiler embryos increased pectoralis major muscle development compared with controls (Xu et al., 2021). These responses were associated with increased expression of genes involved in muscle growth and cell cycle regulation, suggesting that NR enhanced NAD-dependent metabolic pathways regulating myogenesis. Short-term NR supplementation in finishing pigs prior to harvest improved mitochondrial integrity and slowed apoptotic activation in muscle tissue, which contributed to improved pork color stability and postmortem muscle metabolism (Zhu et al., 2025).

Collectively, these findings suggest that NR supplementation can influence muscle metabolism and mitochondrial function in livestock species by increasing NAD+ availability and enhancing oxidative metabolic pathways which could lead to improved growth performance. The present study and others have revealed that there is FCR benefit in an increased NA supplementation but not NR supplementation, implying commercial turkeys might require NA at higher concentration that present recommendations. However, it should be noted that the treatment additions were made on a physical weight basis of either 70 mg NA/kg diet or NR where NA is 123.11 g/mol at 99.5% pure and NR-Cl is 290.7 g/mol resulting in 255.25 g/mol or 88% purity of the NR-Cl product. This implies that there is 2.3 times the amount of NA molecules than NR-Cl molecules per gram of additive which could be indicative of why there

were not FCR differences observed in from the control to the +NR treatment, but were with +NA.

Measures of intestinal morphology such as VH, CD, and V:C are commonly used indicators of intestinal health and absorptive capacity in poultry. Taller villi are indicative of a greater intestinal surface area available for nutrient digestion and absorption. Increased absorptive surface area can enhance the capacity of nutrient uptake, which may translate into improved BWG and FCR. Conversely, crypts are the sites of epithelial cell proliferation. Deeper crypts indicate increased epithelial turnover, which can occur in response to intestinal stress, inflammation, or damage. When CD increases without a corresponding increase in VH, more nutrients may be diverted toward maintaining intestinal tissue rather than supporting growth, which can negatively affect FCR.

Niacin may influence intestinal morphology because it serves as a precursor for the coenzymes NAD+, which are essential for cellular energy metabolism and redox reactions involved in enterocyte proliferation and differentiation. The intestinal epithelium has a high rate of cell turnover, with epithelial cells proliferating in the crypts and migrating along the villi as they mature. Adequate NA supplementation supports ATP production and metabolic activity required for epithelial turnover, which may promote increased VH and reduce excessive CD, thereby improving the V:C and absorptive capacity. Consequently, increasing dietary niacin has the potential to support intestinal epithelial development and nutrient absorption, particularly when niacin supply is limiting or when intestinal tissues are under metabolic or inflammatory stress. In the present study, intestinal morphology observed in the distal ileum revealed no evidence of differences in VH, CD or V:C ($P > 0.325$; Table 4) with supplementation of NA or NR. Although no differences we observed in the experiment reported herein, at d 7 post wean

additional NA at 75, 100, 150 and 250% of the piglet requirement (NRC, 2012) linearly decreased VH and CD. In contrast during the same experiment at d 14 post wean, increasing NA supplementation tended to linearly increase ileum VH and CD in weaned piglets (Yi et al., 2021).

It can be concluded that the improvements due to NA supplementation in FCR in the present study did not come from an improvement in increased absorbative area via improved morphology as suggested in other studies. This likely indicates that the basal diet provided sufficient NA to support normal intestinal epithelial development, leaving limited opportunity for further intestinal morphology improvements with additional supplementation. However, the improvement in FCR observed with additional NA may have resulted from enhanced metabolic efficiency of absorbed nutrients rather than detectable changes in intestinal morphology.

The NRC (1994) suggests the requirement for this age turkey poult is 60 mg NA/kg diet; however, in the present study, it is demonstrated that there is an improvement in FCR when feeding 172 mg NA/kg diet compared to 102 mg NA/kg diet. This is over double the genetic supplier recommendation of 85 mg NA/kg diet, and on the higher end of the range of 105-160 mg NA/kg diet that vitamin suppliers recommend. Further research is needed to expand the NA levels of supplementation in order to observe a break point in FCR improvement and to better understand the economic gain of added NA in turkey hen diets.

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Table 2.1 Experimental basal diet formulation[1]

Item	Pre-starter, %	Starter, %
Corn, 850 µm	42.87	48.82
Soybean meal, 46% CP	48.56	42.87
Limestone	1.44	1.29
Dicalcium phosphate	2.31	2.33
NaCl	0.36	0.36
Choline chloride, 60%	0.20	0.20
L-Lysine HCl	0.26	0.27
DL-Methionine	0.33	0.29
L-Threonine	0.07	0.07
Vitamin mineral premix[2]	0.30	0.30
Phytase[3]	0.02	0.02
Soybean oil[4]	3.29	3.18
Total	100.00	100.00
Calculated composition, %		
Crude protein	28.26	25.95
Tryptophan	0.32	0.29
SID Lys	1.62	1.49
AA Ratios		
Lys:Met	41.32	40.89
Lys:TSAA	65.00	65.00
Lys:Trp	19.48	19.11
Lys:Ile	65.44	64.85
Lys:Val	69.00	69.00
Lys:Arg	107.20	105.59
Lys:Thr	59.00	59.00
Niacin, mg/kg	102.50	102.50
AME, kcal/kg	2953.40	3014.50

[1]Day-old turkey hens (Hybrid Hendrix Genetics, Beatrice, NE; *N* = 900) were randomly allocated to 30 pens located across 10 locations of a research barn (*N* = 30/pen) for a 42-d growth study.
[2]Vitamin mineral premix provided per kg of complete feed, Vit A 16,534 IU, Vit D_3 5,556 IU, Vit E 33 IU, Vit K 5.6 IU, Vit B_1 1.32 mg, Vit B_2 11.24 mg, Vit B_3 102.5 mg, Vit B_5 17.9 mg, Vit B_6 4.0 mg, Vit B_7 0.07 mg, Vit B_9 0.66 mg, Vit B_{12} 0.02 mg; copper sulfate13.95 ppm, iron sulfate 138 ppm, calcium iodate 1.50 ppm, manganese sulfate 105 ppm, zinc sulfate 105, selenium sodium selenite 0.30 ppm
[3]Quantum Blue 10G (AB Vista, Plantation, FL) 10,000 FTU/g
[4]2% of soy oil for each basal diet was applied post pelleting. The remaining difference was added at the mixer prior to pelleting.

Table 2.2 Vitamin and mineral premix recommendations and inclusion in kilograms

	Provided		Recommendations	
Item	Premix[2]	Diet	NRC[3]	Hybrid
Vit A, IU	5,511,463.84	16,530.00	5,000.00	12,500.00
Vit D_3, IU	1,851,851.85	5,554.08	1,000.00	5,000.00
Vit E, IU	11,022.93	33.06	12.00	100.00
Vit K, mg	1,873.90	5.62	1.75	5.00
Vit B_{12}, mg	6.39	0.02	0.003	0.03
Riboflavin, mg	3,747.80	11.24	4.00	15.00
Niacin, mg	34,171.08	102.49	60.00	85.00
Pantothenic acid, mg	5,952.38	17.85	10.00	25.00
Biotin, mg	24.25	0.07	0.25	0.27
Folic acid, mg	220.46	0.66	1.00	3.00
Thiamin, mg	440.92	1.32	2.00	4.00
Pyridoxine, mg	1,322.75	3.97	4.50	6.50
Cu sulfate, mg	4,650.00	13.95	8.00	15.00
Fe sulfate, mg	46,000.00	138.00	80.00	60.00
Ca Iodate, mg	500.00	1.50	0.40	2.00
Mn sulfate, mg	35,000.00	105.00	60.00	100.00
Zn sulfate, mg	35,000.00	105.00	70.00	100.00
Sodium Se, mg	100.00	0.30	0.30	0.30

[1] All values listed on a per kilogram basis

[2] This vitamin and mineral premix did not have any source of choline included and therefore choline chloride was provided through another source.

[3] National Research Council. 1994. Nutrient Requirements of Poultry. 9th rev. ed. Natl. Acad. Press, Washington, DC.

Table 2.3 Supplementation of niacin or nicotinamide riboside on growth performance in commercial turkey hens from 0-42 days of age.

Item	Control[2]	Added NA[3]	Added NR[4]	SEM	*P* <
0 to 18d					
BWG, g	478.9	492.9	475.4	22.82	0.839
FI, g	599.9	591.5	592.4	21.51	0.953
FCR	1.26	1.21	1.25	0.018	0.133
19 to 42d					
BWG, g	1,930.6	2,032.6	1,966.9	40.07	0.210
FI, g	2,754.3	2,862.4	2,755.8	60.73	0.433
FCR	1.43	1.41	1.40	0.010	0.199
0 to 42d					
BWG, g	2,409.5	2,525.5	2,442.3	59.88	0.381
FI, g	3,297.9	3,382.7	3,298.4	87.38	0.727
FCR	1.37[a]	1.34[b]	1.35[ab]	0.007	0.021

[1]At hatch, 900 turkey hens (Commercial Production Stock, Hybrid Hendrix Turkeys, Beatrice, NE) were placed in 30 floor pens (*N* = 30/pen) and 10 replicates per treatment.
[2] The VTM provided 102 mg niacin (NA)/kg diet.
[3] The VTM provided 102 mg NA/kg diet and 70 mg NA/kg diet was added with 2% soybean oil post pellet liquid application to provide a total of 172 mg/kg of niacin (ROVIMIX® Niacin, 99.5%, DSM-Firmenich, Parsippany, NJ, USA)
[4] The VTM provided 102.5 mg NA/kg while 70 mg/kg nicotinamide riboside (NR) was added with 2% soy oil PPLA to provide a total of 102 mg NA/kg and 70 mg/kg NR (NIAGEN®, ChromaDex, Los Angeles, CA).
[ab] Difference in superscript indicates a statistically significant difference of $P \leq 0.05$.

Table 2.4 Effect of added niacin or nicotinamide riboside on ileal histology in turkey hens

Item, µm	Control[2]	Added NA[3]	Added NR	SEM	*P* < value
Villus height	899.2	835.3	866.1	54.89	0.703
Crypt depth	161.3	156.6	216.4	32.53	0.325
V:C	5.59	5.32	5.24	0.312	0.703

[1]At hatch, 900 turkey hens (Commercial Production Stock, Hybrid Hendrix Turkeys, Beatrice, NE) were placed in 30 floor pens (*N* = 30/pen) and 10 replicates per treatment. On d 42 two birds per pen were sacrificed for the collection of ileum sections and sent for hematoxylin and eosin staining (Iowa State University Veterinary Diagnostic Laboratory, Ames, IA). Ten villus and 10 crypts were measured and averaged to obtain a single bird value. Measurements were performed by a technician blinded to treatments.

[2] The VTM provided 102 mg niacin (NA)/kg diet.

[3] The VTM provided 102 mg NA/kg diet and 70 mg NA/kg diet was added with 2% soybean oil post pellet liquid application to provide a total of 172 mg/kg of niacin (ROVIMIX® Niacin, 99.5%, DSM-Firmenich, Parsippany, NJ, USA)

[4] The VTM provided 102.5 mg NA/kg while 70 mg/kg nicotinamide riboside (NR) was added with 2% soy oil PPLA to provide a total of 102 mg NA/kg and 70 mg/kg NR (NIAGEN®, ChromaDex, Los Angeles, CA).

Chapter 3 - Evaluation of dietary riboflavin concentration on growth performance and intestinal permeability of turkey hens raised during summer months.

ABSTRACT

Riboflavin recommendations range from 4 to 21 mg riboflavin/kg of finished feed for turkeys during the first 6 wks of age. Environmental conditions during the summer can challenge turkey production with heat stress negatively impacting body weight gain (BWG), feed intake (FI), and feed conversion ratio (FCR), as well as intestinal integrity. Riboflavin places a crucial role in the antioxidant defense system and cellular energy metabolism. To better understand riboflavin requirements of starter turkey hens during summer months, a study was conducted in July and August to evaluate dietary riboflavin inclusion on growth performance and intestinal permeability. A 43-d study was conducted with dietary riboflavin levels of 11.24, 16.86, 22.48, or 28.10 mg/kg diet to evaluate growth performance and intestinal permeability. No differences were observed in BWG, FI, or FCR from d 1-21. From d 22-43 and overall (d 1-43), there was no effect of dietary riboflavin on BWG or FI, however, FCR tended to quadratically respond ($P = 0.064$) to increasing dietary riboflavin concentrations. There was no response detected in fluorescein isothiocyanate–dextran intestinal permeability with increasing riboflavin inclusion ($P > 0.05$) even after a period of environmental conditions of greater than 32℃ and greater than 50% relative humidity for 5 d. These results suggest that dietary riboflavin inclusion of 11.24 mg/kg diet appears sufficient during the summer months for maintenance of growth performance and intestinal permeability.

INTRODUCTION

Approximately 20 million poults are placed each month during June, July and August to meet the consumption demand of the holiday season in the United States (USDA, 2025). Commercial meat-type turkeys grow rapidly and with high efficiency leading to increased metabolic heat production and leading to risk of environmental heat stress during the summer months. Exposure to elevated temperatures and humidity during the summer months can negatively impact growth and feed efficiency (Abdel-Moneim et al., 2021). High temperature (≥ 29℃) combined with high humidity (≥ 50%) can induce heat stress after 2-6 hrs. of exposure (Fairchild, 2017; Iyasere et al., 2021). In conjunction with depressed growth performance during heat challenges, oxidative stress also occurs and can lead to immune suppression and intestinal integrity disruptions (Ahmad et al., 2022; Ashoori and Saedisomeolia, 2014; Rostagno, 2020).

Some mitigation strategies can alleviate depressed growth and physiological stress during summer months. Producers can manage ventilation to help with cooling, manage feeding schedule to promote eating during cooler hours to not compound metabolic heat during high temperature hours, or add electrolytes in the feed or water to stimulate water intake for cooling purposes. There is also research to support vitamin supplementation (e.g., A, C, E and B-complex) in alleviation of physiological stress symptoms caused by heat stress (Sahin and Kucuk, 2003; Akinyemi and Adewole, 2021). Physiological stress like panting, wing spanning, corticosterone stimulation to suppress appetite, increase reactive species leading to oxidative damager and intestinal barrier disruption can be induced by environmental stress.

The intestinal barrier consists of structural proteins that create tight junctions (**TJ**) between enterocytes, regulating paracellular permeability and preventing uncontrolled movement of nutrients, bacteria and endotoxins from the lumen. While ions (e.g., Na^+, Cl^-, K^+, Ca^{2+}), and

water may pass through TJ; bacteria, and larger molecules (e.g., toxins, undigested proteins) are held to the luminal side of the intestine. Tight junctions act as a protective barrier to the blood stream and body. However, during heat stress, intestinal integrity is compromised due to the loosening of TJ through oxidative stress and inflammatory signaling pathways (Olayiwola, 2025). Compromised intestinal integrity can decrease nutrient absorption and allow bacterial passage which puts turkeys at risk for infections and systemic inflammation, further depressing performance (Awad et al., 2017).

Riboflavin acts in the body as flavin adenine dinucleotide (**FAD**) and flavin mononucleotide (**FMN**) with FAD being a cofactor to multiple redox reactions in the antioxidant defense system. Notably FAD is involved in glutathione reductase, thioredoxin reductase, and the electron transport chain reduction and oxidation, each of which contributes to intracellular antioxidant systems in the enterocyte, epithelial cell proliferation and repair, and tight junction maintenance. Presently there is a wide range of recommendations from 4-21 mg riboflavin/kg diet for optimal environmental conditions. However, there is limited research investigating vitamins in turkeys especially during increased temperatures and humidity. The objective of this study was to investigate the supplementation of riboflavin on growth performance and intestinal permeability when fed to turkey hens during periods of elevated environmental temperatures.

MATERIALS AND METHODS

This experiment was conducted at Kansas State University Poultry Unit in Manhattan, KS, using experimental protocols and methods approved by the Institutional Animal Care and Use Committee (4850-9000).

Animal care and management

One-day-old commercial turkey hens (Hybrid Hendrix Genetics, Beatrice, NE) were placed into 32 pens (*N* = 31 turkeys per pen) within 8 locational blocks balanced by initial BW resulting in 8 replicates per treatment. Treatments consisted of a basal control containing 11.24 mg riboflavin/kg diet, or the basal control plus the addition of riboflavin (ROVIMIX B2 80 SD, DSM-Firmenich, Parsippany, NJ) in 5.62 mg/kg diet increments resulting in dietary treatments of 11.24, 16.86, 22.48, and 28.10 mg riboflavin/kg diet. The basal control was formulated (Table 1) to meet the nutrient requirements provided by the genetic supplier (Hendrix Genetics, 2022) except for vitamins and minerals which were provided through a commercially available vitamin mineral premix (**VTM;** Purina Poultry VTM -P, 750Z, Arden Hills, MN). This specific VTM met the turkey hen NRC (1994) recommendations for vitamins and minerals except biotin, folic acid, thiamine and pyridoxine. All diets were mixed using a 907 kg twin shaft counterpoise mixer (Hayes and Stoltz, Model TRDB63-0152, Fort Worth, TX). Mash diets were then steam conditioned (25 × 140 cm Wenger twin shaft pre-conditioner, Model 150, Sabetha, KS) and pelleted on a 1-ton 30-horsepower pellet mill (1012-2 HD Master Model, California Pellet Mill, Crawfordsville, IN) equipped with a 4.4 × 35 mm (length: diameter 8) pellet die. Prior to pelleting, the conditioner was set for a conditioning retention time of 30 sec. Target conditioning temperature range was 82-85℃.Temperature readings were recorded three times per batch for conditioning temperature and hot pellet temperature quality control. Pellets were cooled using a 1-ton capacity counter flow cooler (OP><FLO™ AIR COOLER, Bliss Industries, Ponca City, OK) for 15 min prior to sampling and bagging. Diets were fed in crumble form utilizing a 23 × 15 cm single pair 3-horsepower roller mill (A24013, RMS, Harrisburg, SD). Diets were fed in two phases of pre-starter from day 0-21 and starter day 22-43 and fed in crumble form.

Poults were housed in floor pens (1.5 × 4.3 m) with unused wood litter shavings, and each pen was equipped with a single hanging feeder (Chore Time, Milford, IN) and a 4-cup drinker system (Lubing EasyLine; Campodarsego, Italy) allowing for *ad libitum* access to feed and water. Feeder trays were placed for the first 3 d to allow stimulation of feed intake (**FI**). The lighting schedule was stair-stepped to 18 h light and 6 h dark from d 0 to 7. Lighting was maintained at 18L:6D at 90 LUX until wk 3 when half of the light bulbs were taken out to reduce LUX. Dark hours were from 10 pm to 4 am for the pre-starter phase and were adjusted to 12 pm to 6 pm as a heat management strategy in the starter phase. The environmental temperature was maintained to follow the genetic supplier recommendations and stair-stepped down from 34℃ to 23℃ over the course of the study. It is worthy to note these conditions were not upheld due to summer months and inability to air condition barns, but ventilation strategies were maximized, and temperatures and relative humidity (**RH**) were recorded across four data loggers (HOBO, Onset Computer Corp., Bourne, MA, USA) twice per day (Table 2). Body weight gain (**BWG**) and FI data were collected for d 0-21 and 21-43 with feed conversion ratio (**FCR**) being calculated for each phase and adjusted for mortality.

Intestinal permeability

Fluorescein isothiocyanate-dextran (**FITC-d**, 4,000 kD, Sigma Aldrich, St. Louis, MO) was utilized as a marker of intestinal permeability. Methodology used followed Baxter et. al. (2017). In preparation for the administration of oral gavage, a solution was prepared to be 8.32 mg FITC-d to 1 mL of distilled water. The mixed solution was protected from ultraviolet exposure and kept in a cooler during transportation and utilization. On d 37, 2 birds per pen were selected to be administered FITC-d oral gavage at 8.32 mg/kg BW. To develop the standard

curve for the FITC-d assay, 8 total turkeys representing 2 turkeys per treatment were randomly selected from 8 pens and received a water gavage that were not already gavaged with FITC-d and bled. Birds were weighed and the appropriate dosage of FITC-d solution or water (1 mL to 1 kg of BW) was administered. Birds were bled approximately 1 h after gavage from the wing-brachial vein (Ison et al., 2019). During this waiting period, birds were restricted from feed and water. Once the blood samples were collected, samples were transported to the laboratory and centrifuged at 1,000 × g for 15 minutes and aliquoted. Samples were stored at -80℃ until analyzed as previously outlined (Baxter et al., 2017).

Statistical analysis

Data were analyzed as a randomized complete block design utilizing the PROC GLIMMIX procedure of SAS (SAS Institute LLC, Cary, NC). The blocking factor was location block in the barn, pen as experimental unit and fixed effect of treatment. Linear and quadratic contrasts were analyzed for the addition of riboflavin. Significant statistical differences were considered $P \leq 0.05$, and statistical tendencies were considered $P < 0.10$.

RESULTS AND DISCUSSION

Average weekly temperatures and relative humidity are reported in Table 2. Daily temperature averages were similar to the optimal recommended temperatures for rearing for the pre-starter phase. In the starter phase, daily temperature lows were greater than the optimal recommended temperature, resulting in daily average temperature being greater than recommended temperatures. During the starter phase, d 33-38, a heat event occurred where daily highs were greater than 32℃ for daily averages and lows being above optimal temperature.

Average daily RH was greater than 50% for the entire trial. It is known that poults develop thermoregulation by 12-14 d of age with brooding temperatures recommended 32-35℃ (Fairchild, 2017). Consistent daily exposure for 7 or more days to temperatures of 30–35℃ combined with moderate RH (> 50%) has shown to induce physiological heat stress responses in broilers within 4-12 h of exposure (Achour et al., 2025; Alhenaky et al., 2017; Awad et al., 2019; Rocchi et al., 2022). There is minimal literature that reveals the minimum time and temperature of exposures in turkeys which induces heat stress. Some studies have utilized cyclical heat stress over several weeks during studies in turkeys in controlled environments. However, the present study is more applicable to commercial scenarios due to the unforced nature of the heat challenge. Poults in the present study experienced temperatures exceeding 32℃ for approximately 6 h/d for 6 d in a row, study d 33–38, with RH greater than 50%, and were observed to be panted and wing spanning during mid-day checks. This is indicative of environmental conditions that may have imposed physiological heat challenge during this period.

From d 0 to 21, there was no evidence of differences ($P > 0.29$) in BWG, FI, or FCR when turkeys were fed diets with increasing concentrations of added riboflavin from 11 to 28 mg riboflavin/kg diet (Table 3). From d 22 to 43, there was no evidence of differences ($P > 0.12$) in BWG or FI when turkeys were fed diets with increasing concentrations of dietary riboflavin. There was a tendency (quadratic, $P = 0.06$) for turkeys fed up to 22.5 mg riboflavin/kg diet to have improved FCR. Overall, d 0-43, there was no evidence of differences ($P > 0.17$) in BWG and FI between turkeys fed increasing concentrations of dietary riboflavin. Regardless, the quadratic improvement in FCR tended to remain (quadratic, $P = 0.09$) for turkeys fed up to 22.5 mg added riboflavin/kg diet.

Vitamin suppliers recommend 15-20 mg riboflavin/kg diet for turkey poults from 1-6 wks of age (DSM-Firmenich, 2022). These levels are above the recommendation of 3.5 mg riboflavin/kg diet (NRC, 1994) but similar to the genetic supplier's recommendation of 10-18 mg riboflavin/kg diet (Hendrix Genetics, 2022). The present data compliment previous studies that determined 3.5 mg riboflavin/kg diet would maximize growth performance in 21 d old poults (Ruiz and Harms, 1989) or 4.6-5.4 mg riboflavin/kg diet would maximize growth performance from 0-56 d in modern genetic poults (Thesing et al., 2023). The present study observed no difference in BWG and FI above 11.2 mg riboflavin/kg diet which was the lowest inclusion level. Riboflavin functions as a precursor to FAD and FMN, which are cofactors in oxidative metabolism and mitochondrial energy production. When riboflavin supply is adequate to support these metabolic processes, additional supplementation may not further improve growth performance. This implies poults were not heat challenged during the pre-starter phase or that riboflavin levels were adequate. However, during the starter phase, d 22-43, poults were challenged with increase temperatures at moderate RH (> 50%) which revealed a tendency for improved FCR at 22.5 mg added riboflavin/kg diet suggests that modest increases in riboflavin could enhance feed efficiency without affecting FI or growth performance. Therefore, the present data suggests that there may be an increased requirement for riboflavin supplementation when turkey poults in the starter phase are subject to acute heat challenges to optimize growth performance.

On d 37 of the study, 2 birds per pen were randomly selected to receive oral gavage of FITC-d to assess intestinal permeability. The 5 d prior to FITC-d gavage, birds were naturally heat challenged at 32℃ or greater and 50% or more RH for at least 6 h per d (Table 2). Regardless, there was no evidence of a response to increasing riboflavin supplementation on

FITC-d permeability in the turkey hens ($P > 0.05$). Previous literature supports an increase in serum endotoxin lipopolysaccharide translocation through the intestinal barrier in broilers when submitted to acute heat stress for 10 d (35℃ for 4 hr/d; Alhenaky et al., 2017). In addition, Ruff et al. (2020), reported an increase in FITC-d serum concentration when broilers were continually heat stressed (35℃) from d 21 to d 35 or 42. Therefore, heat stress challenges in broilers have shown an increase in serum levels of FITC-d or lipopolysaccharide suggesting that tight junctions may have been disrupted. There is no previous data measuring the implications of heat challenge on intestinal permeability in turkeys.

In the present study, there was not cyclic heat stress subjected to the turkey hens, but environmental acute heat challenges were recorded (Table 2). The relatively short duration of elevated temperatures compared with previous studies may have limited the magnitude of intestinal barrier disruption. As a result, the birds may not have experienced sufficient intestinal permeability changes for dietary riboflavin supplementation to influence FITC-d translocation. Additionally, the absence of BWG and FI differences between treatments suggests that birds maintained normal physiological function despite the temporary heat challenge conditions. If intestinal integrity was not severely compromised, the ability of riboflavin to mitigate oxidative or metabolic stress in intestinal tissues may not have been observable through FITC-d measurements. Therefore, under the environmental conditions observed in this study, supplementing riboflavin above genetic and vitamin supplier levels (12.5-21 mg/kg) did not further improve growth performance or intestinal permeability in turkey hens.

Overall, the hens had the best FCR when 22.5 mg riboflavin/kg diet was added through a VTM premix, but no further benefit up to 28 mg riboflavin/kg diet, demonstrating there is no need for additional supplementation beyond the already published recommendations from the

genetic and vitamin suppliers (12.5-21 mg riboflavin/kg diet). Considerations should be taken in that birds in the present study were not tested in a commercial setting, where there would be a higher stocking density and dirty litter. This study did not stock at commercial housing densities therefore compounding stressors with high heat and humidity that would be present in commercial barns were absent in this study. Due to T and RH not being induced but rather dependent on weather, birds were exposed to warm days but ultimately had the opportunity to recover from the heat during cooler nights. Therefore, further research is needed to better understand the practical implication of riboflavin supplementation in commercial conditions.

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Table 3.1 Diet composition (as-is basis)

Ingredient, %	Pre-starter	Starter
Corn, 650 μm	42.86	48.82
Soybean meal, 46% CP	48.56	42.87
Dicalcium phosphate	2.31	2.33
Limestone	1.44	1.29
NaCl	0.36	0.36
L-Lysine	0.26	0.27
DL-Methionine	0.33	0.29
L-Threonine	0.07	0.07
Vitamin mineral premix[2]	0.30	0.30
Choline chloride, 60%	0.20	0.20
Phytase[3]	0.02	0.02
Soybean oil	3.29	3.18
TOTAL	100	100
Calculated composition[4]		
Crude protein, %	28.26	25.95
SID Lys, %	1.62	1.49
SID Met, %	0.67	0.61
AME, kcal/kg	2,953	3,042

[1] Basal diet formulation met or exceeded the Hybrid recommendations for the commercial hen. The basal diet was mixed and divided into four equal lots to be additionally mixed with treatment additions of riboflavin to achieve 11.24, 16.86, 22.48, and 28.10 mg/kg (ROVIMIX B2 80 SD, DSM-Firmenich, Parsippany, NJ)

[2] Provided per kg of complete feed, Vit A 16,534 IU, Vit D_3 5,556 IU, Vit E 33 IU, Vit K 5.6 IU, Vit B_1 1.32 mg, Vit B_2 11.24 mg, Vit B_3 102.5 mg, Vit B_5 17.9 mg, Vit B_6 4.0 mg, Vit B_7 0.07 mg, Vit B_9 0.66 mg, Vit B_{12} 0.02 mg

[3] Quantum Blue 10G (AB Vista, Plantation, FL) 10,000 FTU/g feed additive

[4] SID-standardized ileal digestible; AME-apparent metabolizable energy

Table 3.2 Average weekly temperatures and relative humidity by phase

		Temperature[2], ℃			Relative Humidity[2], %
	Optimal[3]	Average	Low	High	Average
Week					
d 0-7	32.2	31.1	28.3	35.0	58
d 8-14	28.9	30.0	25.0	35.6	62
d 15-21	27.8	26.1	22.8	29.4	67
d 22-28	25.6	27.8	25.6	30.6	72
d 29-35	23.3	28.9	26.7	31.1	64
d 36-42	21.1	28.3	25.6	31.1	64

[1] 960 hatched Hybrid commercial hens were allotted to 8 locational blocks balanced by BW. Hens were fed one of four dietary treatments for 43 days, with BWG, FI, and FCR ratio measured.

[2] Temperature and relative humidity were recorded twice daily across four HOBO data loggers (Onset Computer Corp., Bourne, MA, USA) with daily temperature and RH averaged and low and high reported.

[3] Optimal temperature was the recommended barn temperature for the age of turkey hen set forth by the genetic supplier for optimal growth.

Table 3.3 Growth performance and intestinal permeability in turkey poults fed varying levels of riboflavin.[1]

	[2]Riboflavin addition, mg/kg					Probability, *P* <	
Item	11.2	16.9	22.5	28.1	SEM	Linear	Quadratic
D 0-21							
BWG, g	616	627	622	628	11.04	0.406	0.478
FI, g	780	786	797	800	15.80	0.286	0.866
FCR	1.31	1.32	1.31	1.31	0.008	0.452	0.429
D 22-43							
BWG, g	1,927	1,876	1,899	1,958	20.55	0.118	0.619
FI, g	2,824	2,786	2,770	2,855	34.62	0.597	0.596
FCR	1.47	1.48	1.46	1.46	0.010	0.186	0.064
D 0-43							
BWG, g	2,542	2,503	2,521	2,586	29.96	0.172	0.938
FI, g	3,573	3,527	3,549	3,627	47.45	0.374	0.953
FCR	1.43	1.44	1.42	1.42	0.008	0.180	0.089
FITC-d[4] µm/mL	0.37	0.30	0.33	0.33	0.08	0.778	0.692

[1] 960 hatched Hybrid commercial hens were allotted to 8 locational blocks balanced by BW. Hens were fed one of four dietary treatments for 43 days, with body weight gain (BWG), feed intake (FI), and feed efficiency (FCR) ratio measured. On d 37 birds were weighed and 2 random birds per pen were selected for an oral gavage of fluorescein isothiocyanate dextran to observe intestinal permeability.

[2]Dietary treatments consisted of a dose dependent increase in riboflavin (ROVIMIX B2 80 SD, DSM-Firmenich, Parsippany, NJ)

[3]Data were analyzed as a randomized complete block design utilizing PROC GLIMMIX procedure of SAS (9.4, Cary, NC). Linear and quadradic contrasts were tested. Results were considered significant at P ≤ 0.05.

[4] Fluorescein isothiocyanate-dextran

[ab] Means within a row lacking a common superscript letter are different ($P < 0.05$).

Chapter 4 - Impact of sorghum particle size on feed characteristics, growth performance, gizzard weight and pH in broiler chickens

ABSTRACT

Sorghum is a cereal grain that can serve as an energy source in broiler diets when it is competitively priced for feed formulation. However, there is limited data on the optimal particle size to grind sorghum for broiler diets. The objective of this study was to evaluate sorghum particle size effects on growth performance, gizzard weight, and pH in broilers from 4-49 d of age. A 49-d floor pen study was conducted with a treatment structure of 850 µm for corn and 414, 606, 821, 1046, and 1124 µm for the sorghum treatments. Body weight gain (**BWG**), feed intake (**FI**), and feed conversion ratio (**FCR**) adjusted for mortality d 4-49. On d 49, two birds of average body weight were selected from each pen for determination of gizzard pH and weight. Overall (d 4-49) birds fed sorghum-based diets had increased ($P < 0.05$) BWG and FI compared to those fed the corn-based diets, resulting in no difference in FCR (P > 0.05?). From d 4-49 increasing sorghum particle size tended to increase FI (linear, $P = 0.059$) and BWG (quadratic, $P = 0.056$) of broilers, with the majority of the improvements in BWG occurring when sorghum particle size was ground to 1,200 µm. In conclusion, broilers fed the sorghum-based diets had increased BWG and FI compared to those fed the corn-based diets with no difference observed in FCR. Based on the design of this experiment, targeting a coarser particle size in the 1,000 to 1,200 µm range was optimal for growth performance and gizzard development.

INTRODUCTION

Sorghum is a widely utilized alternative to corn in broiler diets, particularly in regions where it is readily available or cost-effective. Although the nutritional content of sorghum is similar to that of corn, sorghum traditionally contains tannins which affect nutritional properties. High tannin-containing sorghum has been shown to reduce broiler growth performance due to decreased nutrient digestibility whereas low-tannin or tannin-free sorghum have been reported to have similar feeding values to that of corn, resulting in improved growth performance compared to high tannin sorghum (Selle et al., 2010; Moritz et al., 2022). In 2025 a total of 91 sorghum samples from the U.S. were tested and confirmed to have less than 4.0 mg catechin equivalents (CE)/g, implying an absence of tannins (U.S. Grains Council 2025), which is consistent with tannin survey results from 2023 and 2024 (U.S. Grains Council 202).

Grain particle size (geometric mean diameter; dgw) influences the development and physiology of the digestive tract. Specifically, the gizzard is responsible for mechanical grinding and the initiation of nutrient digestion and is influenced by the physical characteristics of the diet, such as measured by dgw. A more active gizzard is typically associated with coarser feed, often exhibits a lower pH which may contribute to improved pathogen control and nutrient digestibility. Coarser corn particles (e.g., > 600 µm) enhance gizzard muscularity and have been linked to increased retention time and lower digesta pH (Xu et al., 2015; Naderinejad et al., 2016). Previous research has suggested that dietary dgw modulates gizzard pH, likely due to its effects on fermentation activity and acid secretion (Ghasemi-Aghgonbad et al., 2024). On the other hand, finely ground particles reduce gizzard activity, increase digesta pH, and accelerate passage rate, potentially limiting nutrient absorption (Svihus, 2011).

Original research on sorghum particle size determined that grinding sorghum to a dgw of 300 µm optimized feed conversion ratio (FCR) in broilers (Healy et al., 1991). However, recent research has determined that whole sorghum can be added to broiler diets with no negative impacts on growth performance compared to ground sorghum or corn (Fernandes et al., 2013). Limited research on the effects of sorghum particle size in broilers has been reported. Similar to the response of feeding coarsely ground corn, it is hypothesized that there would be no negative impact of increasing sorghum dgw (e.g., S400-S1200 µm) on growth performance and that gizzard weight would increase and gizzard contents pH would decrease with increasing sorghum particle size. The objective of this study was influence of sorghum dgw on growth performance, gizzard weight and gizzard digesta pH in broiler chickens.

MATERIALS AND METHODS

This experiment was conducted at the Kansas State University Poultry Unit in Manhattan, KS, using experimental protocols and methods approved by the Institutional Animal Care and Use Committee (4850-29520).

Animal care and management

A total of 864-day old by-product breeders (Cobb-Vantress, Siloam Springs, AR) were allotted to 72 pens (n = 12 broilers per pen) and placed on a common diet for the 4 d. On d 4, pens were reduced to 10 broilers per pen (96.5 × 33 cm) with chicks maintained in an environmentally control room with *ad libitum* access to feed (Chore Time, Milford, IN) and water (Chore-Time Steadi-FLOW, Milford, IN). Dietary treatments were randomly assigned to pens within location block and balanced for average body weight. Body weight gain **(BWG)** and

feed intake **(FI)** data were collected for d 4-12, 13-28, 29-39, and 40-49 with feed conversion ratio **(FCR)** being calculated for each phase after adjustment for mortality. Light to dark period was set to follow industry recommendation of stair stepping from 24 h light at d 0 to 18 h light by d 6, adding 1 h of dark each day until achieving 18 h light and 6 h dark from d 6-49 of the trial.

On d 49, average pen body weight (BW) was calculated, and 2 average weight broilers were selected from each pen and euthanized via CO_2 asphyxiation for the collection of gizzard content acidity (**pH**) and relative gizzard weight (**RGW**). Gizzard contents pH was measured utilizing a portable pH and temperature meter (HI99165, Hanna Instruments, Woonsocket, RI) prior to cleaning out the organ. The probe was inserted in such a manner that it was ensured to not be touching any organ wall while measuring the digesta pH. After pH was determined, the gizzard was trimmed of excess connective tissue, gizzard contents were removed, and gizzards were rinsed with distilled water. Gizzards were weighed, and relative gizzard weights were reported as a percent of BW.

Study design and particle size reduction

A single lot of corn and sorghum were sourced and used for the experiment. Grains were analyzed for mycotoxins, proximate analysis, and amino acid profiles (Table 1). Deoxynivalenol, aflatoxin and fumonisin were analyzed at a commercial laboratory (ATC Scientific, North Little Rock, AR) where samples were extracted and isolated with an immunoaffinity column and analyzed on an HPLC-MS/MS. Proximate analysis and amino acid profiles were completed for whole grain samples (method 982.30 E [a, b, c]; AOAC, 2019), at a commercial laboratory as well (Agricultural Experiment Station Laboratories, University of Missouri, Columbia, MO).

Proximate analysis included dry matter (method 930.15; AOAC, 2019), ash (method 942.05; AOAC, 2019), crude fiber (method 978.10; AOAC, 2019), and crude fat (method 920.39; AOAC, 2019). Nitrogen was analyzed using the combustion method in a LECO analyzer (method 990.03; AOAC, 2019) with crude protein calculated as N × 6.25.

Dietary treatments consisted of a corn-based (**C**; 800 µm) control diet and sorghum-based (**S**) diets with varying sorghum d_{gw} (400, 600, 800, 1000, and 1200 µm). Target grain particle sizes were achieved using a 25-horsepower hammermill (Model 22115, Bliss Industries LLC. Ponca City, OK) equipped with 24 hammers. The chamber diameter measured 56 cm wide with a depth of 29 cm. The hammermill was equipped with a variable frequency drive (**VFD**), resulting in 3,560 rpm when operating at 100%.

To achieve each target d_{gw}, the hammer mill was set to a constant feeder rate of 15%. The hammer mill motor was equipped with a VFD which acts on the motor to slow the speed at which it rotates the shaft that turns the hammers to directly affect tip speed. In brief, a known corn and sorghum particle size setting was utilized from previous research as the baseline settings for each grain. From there, screen size and VFD were manipulated to achieve target d_{gw}. Screen sizes utilized, VFD settings, and calculated tip speeds are recorded in Table 2 with the resulting d_{gw} and geometric standard deviation ($\mathbf{S_{gw}}$). All ground grains needed for the experiment were produced in a single continuous run per treatment to ensure consistency of particle size throughout all dietary phases.

The d_{gw} and S_{gw} were determined according to the American Society of Agricultural and Biological Engineers (method S319.4, ASABE, 2008). Analyses were conducted utilizing the 13-sieve stack with agitators and sieve agent, for 10 minutes in a sieve shaker (Ro-Tap. ASAE S319.4 FEB 2008 R2012). In addition, flowability characteristics were measured using

methodology outlined by Kalivoda et al. (2017). Flowability parameters of ground grains were measured in duplicate using compressibility, critical orifice diameter **(COD),** and angle of repose **(AoR)**, and these values were utilized to calculate the composite flow index **(CFI)**.

Complete diet manufacturing

All feed was manufactured at O. H. Kruse Feed Mill (Manhattan, KS). Diets were formulated to meet or exceed the recommendations set forth by Cobb Vantress for all nutrients (Cobb, 2022). Diet formulations were balanced by standardized ileal digestible lysine, methionine, threonine, valine, calcium, and phosphorus. During formulation, sorghum and corn were assumed to be equal in metabolizable energy in order to keep added fat concentration the same between treatments. Diets were fed as starter (d 4-12), grower-1 (d 13-28), grower-2 (d 29-39), and finisher-1 (d 40-49). All treatments were mixed in a single batch utilizing a 907 kg twin shaft counterpoise mixer (Hayes and Stoltz, Model TRDB63-0152, Fort Worth, TX) and mash diets were steam conditioned (25 × 140 cm. Wenger twin staff pre-conditioner, Model 150) and pelleted on a 1-ton 30-horsepower pellet mill (1012-2 HD Master Model, California Pellet Mill, Crawfordsville, IN) equipped with a 4.4 × 35 mm (length: diameter 8) pellet die. Prior to pelleting, the conditioner was set for an average conditioning retention time of 30 seconds. Pelleting production rate was 14.96 kg/min. Target conditioning temperature range was 82-85℃. Temperature readings were recorded three times per batch for conditioning temperature and hot pellet temperature for quality control. Pellets were cooled using a 1-ton capacity counter flow cooler (OP><FLO™ AIR COOLER, Bliss Industries, Ponca City, OK) for 15 minutes prior to sampling and bagging. Starter diets were crumbled utilizing a 23 × 15 cm single pair 3-horsepower roller mill (A24013, RMS, Harrisburg, SD). Grower-1, grower-2, and finisher-1 diets were fed as whole pellets. Cooled pellet samples were analyzed for pellet durability index

(PDI) in triplicate for each batch of feed. The PDI was measured using a pellet durability tester (Holeman 100, Tekpro Limited, North Walsham, Norfolk, UK) set to 60 sec on all treatments for all phases.

Statistical Analysis

Growth performance and gizzard data were analyzed as a randomized complete block design utilizing the MIXED procedure of SAS 9.4 (SAS Institute LLC, Cary, NC). Dietary treatment was considered the fixed effect, and pen location was the blocking factor. Dunnet's test was used to compare each sorghum treatment back to the corn control, and linear and quadratic contrast were utilized to determine the response to changing sorghum particle size. Results were considered significant at $P < 0.05$, and tendencies between $0.05 < P < 0.10$.

RESULTS AND DISCUSSION

Feed quality parameters

The d_{gw}, S_{gw}, particle size distribution, and flow ability metrics are reported in Table 3. The d_{gw} of all treatments, except for S1,200, were within ± 50µm of the target d_{gw}. The S1200 treatment resulted in a measured d_{gw} of 1,124 µm. The S_{gw} for C800, S400, S600, S800, S1,000, S1,200 were 2.89, 2.89, 2.60, 2.69, 2.46, and 2.66 respectively. The S_{gw} represents the geometric distribution of different particles across screens in the sieve stack. Greater S_{gw} indicates a wider distribution of particle sizes, and a smaller S_{gw} indicates a tighter distribution. The sorghum 800 µm treatment had an S_{gw} of 2.69 compared to the corn S_{gw} of 2.89. The smaller sorghum S_{gw} resulted from less coarse particles (3.48% > 2360 µm) compared to that of corn (11.62% > 2360 µm). As the sorghum d_{gw} increased from 400 to 1000 µm, the S_{gw} decreased from 2.89 to 2.46.

As the sorghum was ground to a smaller particle size, this resulted in more fines in the pan of the sieve stack which led to an increase in S_{gw}. The S_{gw} of the sorghum ground to 1200 µm increased compared to the 1100 µm sorghum. This increase from 1100 to 1200 µm can be explained by a reduction of tip speed during grinding which is known to increase S_{gw} (Pfost, 1976). There was increase in coarse particles for the 1200 µm (13.06% > 2360 µm and 2.29% > 3350 µm), while still having 4.09% fine particles in the pan compared to the 1000 µm (8.35% > 2360 µm; 0.99% > 3350 µm; 3.18% in pan) sorghum.

Flowability is the ability of granular solids and powders to flow during discharge from transportation or storage containments. Poor flowability may result in several types of flow problems within the feed mill that can occur in the bin or silo including bridging, or the formation of an arch that prevents ingredients from flowing out. When producing pelleted complete diets, flowability issues may occur during the storage and handling of ground ingredients; however, the pelleting process largely mitigates flowability concerns in the finished complete diet. The flowability characteristics analyzed included: AoR, compressibility, COD, and CFI (Table 3). In feed manufacturing, AoR is the most common measurement used to evaluate flowability. Ground ingredients with a low AoR represent those that flow more easily, while those with a high angle represent those that are more cohesive and prone to bridging or clogging. This measurement is important for ingredient storage in bins, as it helps predict how a feed ingredient will behave during bin filling and discharge. Knowing the AOR ensures proper hopper slope when choosing bins, prevents feed buildup when considered, and maintains consistent feed delivery to mixers or feeders when concepts are applied correctly. The ground grains for this study numerically observed the best AoR at S1200 (~40°) and worst at S400 (~49°) with all other treatments intermediate (S600, S800, C800, S1000; ~45°), as expected as

the d_{gw} increases, AoR decreases. These data agree with previous studies that analyzed AoR of increasing d_{gw} of corn (Jadhav et al., 2017) and sorghum (Friesen et al., 2024). With the resulting AoR values, S400 would be considered poor flowing material (46.0-55.9º). For C800, S600, S800, S1000, the AoR value decreased to a point that would characterize the ground sorghum as a passable flowing material (41.0-45.9º). The S1200 resulted in the lowest AoR and is characterized as being fair flowing material (36.0-40.9º).

Compressibility measures the change in volume of the sample when increasing levels of compressive force are applied and is influenced by factors including particle size distribution, particle shape, and particle texture (Freeman Technology, 2015). The compressibility of the S1200 treatment was classified as having a fair flow characteristic (16-20%), while the remaining ground grains were characterized as having a passable flow characteristic (21-25%). As sorghum d_{gw} increased, compressibility decreased which is in agreeance with a previous study that observed increasing d_{gw} to have decreasing compressibility (Braun et al. 2021).

The COD is determined using a powder flow ability test instrument (Flodex Model WG-0110, Paul N. Gardner Company, Inc., Pompano Beach, FL). The resulting COD is the size of the smallest orifice in a base plate disc through which the material in a cylinder will discharge. The smallest disk for the C800 and S400 grains would flow through were a 25- and 27-mm diameter hole, respectively, while the remaining ground sorghum would flow through an 18-22 mm diameter hole. The CFI is calculated by combining the results for AoR, compressibility, and COD. The S1200 treatment was the only treatment that resulted in a CFI flow description as fair (60-75). All remaining treatments were classified as passable (45-60).

All diets were steam conditioned for an average conditioning retention time of 30 sec and a conditioning temperature range of 82.4-84.1℃. Average conditioning temperatures and hot pellet

temperatures are reported in Table 4. Pellet durability was greatest in the starter diets and lowest in the finisher diets, with PDI decreasing from 70% in the starter phase to 42%, 29%, and 24% in the grower I, grower II, and finisher phases, respectively, when averaged across all treatments. When averaged across feeding phases, PDI values were 45%, 43%, 40%, 38%, 40%, and 41% for C800, S400, S600, S800, S1000, and S1200, respectively. Overall, PDI declined as feeding phase advanced, and no consistent treatment-related trends were observed across phases.

Growth performance

From d 4-12, there were no differences ($P > 0.05$) in BWG or FI for broilers fed the corn-based control diet compared to birds fed any of the sorghum-based diets. Broilers fed S400 had poorer ($P < 0.05$) FCR compared to those fed C800. There was no evidence of differences in broiler BWG or FCR ($P > 0.240$) when fed sorghum-based diets with varying sorghum d_{gw}. Increasing sorghum d_{gw} resulted in a tendency for decreased (quadratic, $P = 0.085$) FI, with broilers fed S400 having the greatest intake, and broilers fed the remaining treatments having similar FI.

From d 4-28, there were no evidence of differences ($P > 0.05$) in BWG, FI, or FCR for broilers fed the corn-based control diet compared to those fed any of the sorghum-based diets, supporting previous reports that modern, tannin-free sorghum can nutritionally replace corn without compromising broiler performance (Selle et al., 2010; Silva et al., 2017). In addition, there were no evidence of differences ($P > 0.229$) in BWG or FI when broilers were fed sorghum-based diets with varying sorghum d_{gw}. Broilers fed diets with increasing sorghum d_{gw} had poorer FCR (linear, $P = 0.044$). In contrast, Healy et al. (1991) reported improved feed efficiency with decreasing grain particle size in corn-based diets suggesting that finer particles

may enhance nutrient accessibility. The discrepancy between studies may reflect differences in grain type, feed form, or genetic progress in broilers over time.

From d 4-39, BWG of broilers fed the sorghum-based diets were greater ($P < 0.05$) than those fed the corn diets except for those fed the S1000. Broilers fed S1200 had increased ($P < 0.05$) FI compared to broilers fed the corn diets and broilers fed S400 had a tendency for increased ($P < 0.10$) FI compared to those fed the corn-based diets. Broilers fed the S400, S600, S1200 diets had improved ($P < 0.05$) FCR and broilers fed S800 had a tendency for improved ($P < 0.10$) FCR compared to those fed the corn-based diet. Broilers fed the S400 and S1200 diets had increased (quadratic, $P = 0.013$) BWG and FI compared to birds fed the remaining sorghum diets. Broilers fed diets with increasing sorghum d_{gw} had poorer (linear, $P = 0.029$) FCR. These data suggest that performance responses to increasing sorghum particle size during this period were driven primarily by changes in FI rather than improvements in FCR.

From d 4-49, overall BWG and FI were greater ($P < 0.05$) in broilers fed sorghum-based diets compared to those fed the corn-based diets. There was no evidence of differences ($P > 0.05$) in FCR between broilers fed the sorghum and corn-based diets. Broilers fed diets with increasing sorghum particle size tended to increase (linear, $P = 0.059$) FI and BWG (quadratic, $P = 0.056$), with the majority of the improvements in BWG occurring when sorghum d_{gw} was increased from 1000 to 1200 µm. There was no evidence of differences ($P = 0.144$) in FCR of broilers fed diets with varying sorghum d_{gw}.

These findings are consistent with others who reported no differences in BWG, FI, or FCR in broilers fed sorghum ground to 447 or 744 µm from d 11 to 35 (Rodgers et al., 2012), 650 or 850 µm from d 1 to 42 (Silva et al., 2017), and 780–1400 µm (Selle et al., 2017). Collectively, these studies suggest that broilers tolerate a broad range of sorghum particle sizes

without major impacts on overall performance. The present study extends these findings by demonstrating that larger particle sizes (≥1000 μm) may increase FI and late-phase BWG, although improvements in FCR were not consistent.

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Gizzard relative weight and pH

Broilers fed S400 had lower RGW compared with C800 ($P < 0.05$), whereas S1000 increased gizzard weight relative to corn ($P < 0.05$). Within sorghum diets, increasing d_{gw} up to 1000 μm resulted in a quadratic increase in gizzard weight and RGW ($P < 0.01$), indicating enhanced mechanical stimulation with coarser particles. These findings align with previous reports demonstrating that increased dietary particle size stimulates gizzard development measured by organ weight and RGW (Jacobs et al., 2010; Selle et al., 2016).

Gizzard pH was higher in broilers fed S400 compared with corn-based diets ($P < 0.05$) and increasing sorghum d_{gw} tended to decrease gizzard pH (quadratic, $P = 0.06$). This response is consistent with the concept that coarse particles promote increased gizzard muscular activity and gastric acid secretion, resulting in lower luminal pH (Xu et al., 2015; Naderinejad et al., 2016). Similarly, Jacobs et al. (2010) reported lower gizzard pH and increased RGW with coarser corn particle sizes. In contrast, Selle et al. (2016) observed increases in gizzard weight with increasing sorghum particle size but did not detect differences in pH, suggesting that pH responses may depend on grain characteristics.

Although increasing sorghum d_{gw} clearly enhanced gizzard development and tended to reduce gizzard pH in the present study, these physiological changes were not consistently associated with improved FCR. This contrasts with the framework that improved gizzard function enhances nutrient digestibility and feed efficiency (Zaefarian et al., 2016). In the present study, during a 49

d trial sorghum-based diets supported broiler growth performance equal to or greater than a corn-based diet, with improvements in BWG largely driven by increased FI rather changes in FCR. Increasing sorghum particle size influenced FI patterns and gizzard development more than overall FCR, as growth performance maintained with coarser sorghum (≥ 1000 µm). These responses have feed mill implications in that reducing particle size increases grinding energy consumption, decreases hammermill throughput, and accelerates wear on screens and hammers. Particle size reduction is one of the most energy-intensive steps in feed manufacturing, and energy requirement increases exponentially as target particle size decreases. In contrast, coarser grinding improves mill capacity and typically reduces electrical cost per ton (Pfost, 1976). Given that coarser sorghum did not impair overall FCR in the present study, these findings suggest that grinding sorghum to moderate-to-coarse particle sizes may provide an opportunity to reduce processing cost without sacrificing broiler performance.

Sorghum can replace corn in broiler diets without compromising growth performance through market age. In the present study, broilers fed sorghum-based diets supported equal or greater BWG compared with corn, demonstrating that modern tannin-free sorghum is a nutritionally viable primary energy source in commercial broiler production. Importantly, grinding sorghum to coarser particle sizes (≥ 1,000 µm) enhanced gizzard development while maintaining overall feed efficiency, indicating that moderate-to-coarse grinding does not impair nutrient utilization. Because particle size reduction is one of the most energy-intensive steps in feed manufacturing, targeting coarser sorghum particle sizes can substantially reduce grinding energy consumption, increase hammermill throughput, and lower equipment wear, thereby decreasing cost per ton of manufactured feed.

Collectively, these findings demonstrate both biological and economic advantages of utilizing sorghum in the broiler diet. When sourced competitively, sorghum offers an opportunity to reduce ingredient cost, maintain broiler performance, stimulate gastrointestinal development, and improve feed mill efficiency through reduced grinding intensity. Thus, sorghum represents not merely an alternative grain to corn, but a strategically advantageous ingredient when particle size is optimized. Future research should evaluate sorghum ground beyond 1,000 µm under commercial conditions, with specific emphasis on the proportion of whole berries, upper particle size limits that maintain pellet quality, and the practical boundaries of coarse grinding achievable in commercial feed mills.

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Table 4.1 Analyzed composition of cereal grains (as-is basis

Item	Corn	Sorghum
Mycotoxins		
Aflatoxin, ppb	< 2.0	< 2.0
Deoxynivalenol, ppm	0.11	< 0.10
Fumonisin, ppm	0.91	< 0.10
Proximate, %		
Moisture	10.25	12.25
Crude protein	7.79	9.35
Crude Fat	4.38	3.08
Crude Fiber	1.90	1.63
Ash	1.19	1.30
Indispensable AA, %		
Arginine	0.35	0.36
Histidine	0.23	0.25
Isoleucine	0.29	0.39
Leucine	0.92	1.26
Lysine	0.26	0.27
Methionine	0.18	0.17
Phenylalanine	0.38	0.50
Threonine	0.28	0.30
Tryptophan	0.05	0.06
Valine	0.37	0.49
Dispensable AA, %		
Alanine	0.58	0.87
Aspartic acid	0.54	0.64
Cysteine	0.18	0.18
Glutamic acid	1.46	2.04
Glycine	0.18	0.31
Serine	0.36	0.39
Tyrosine	0.22	0.29
Total AA	7.73	9.74

[1] Grain samples were collected upon receiving and sent to be analyzed for mycotoxins (aflatoxin, deoxynivalenol, and fumonisin) at ATC Scientific and proximate and amino acid analysis at the Agricultural Experiment Station Laboratories, at the University of Missouri-Columbia.

Table 4.2 Hammer mill settings and subsequent geometric mean diameter (d_{gw}) and geometric standard deviation (Sgw) when grinding corn and sorghum[1]

				Screen size		
Cereal Grain	Target	d_{gw} (µm)	S_{gw} (µm)	Up (mm)	Down (mm)	Tip Speed (ft/min)
Sorghum	400	414	2.89	4.4	4.4	16,400
Sorghum	600	606	2.60	6.8	5.2	13,325
Sorghum	800	821	2.69	6.8	5.2	9,225
Sorghum	1000	1046	2.46	6.8	4.4	8,610
Sorghum	1200	1124	2.66	6.8	4.4	7,175
Corn	800	850	2.89	9.5	9.5	10,250

[1] Target grain particle sizes were achieved using a 25-horsepower hammermill (Model 22115, Bliss Industries LLC., Pona City, OK) equipped with a variable frequency drive for a 100% tip speed of 20,500 ft/min. The d_{gw} and S_{gw} were determined according to the American Society of Agricultural and Biological Engineers (method S319.4, ASABE, 2008).

Table 4.3 Analyzed physical analysis of ground cereal grains

	Corn	Sorghum				
Item, %	800	400	600	800	1000	1200
Particle size[1]						
d_{gw}	850	414	606	821	1046	1124
S_{gw}	2.89	2.89	2.60	2.69	2.46	2.66
Retained on screen, %						
3350 µm	3.15	0.00	0.00	0.30	0.99	2.29
2360 µm	8.47	0.20	0.90	3.18	7.36	10.77
1700 µm	14.58	1.76	5.51	11.92	17.79	21.14
1180 µm	20.10	11.17	18.94	32.08	34.39	34.50
850 µm	15.76	18.02	21.24	20.26	16.80	12.66
600 µm	11.23	16.55	16.03	9.53	7.26	4.49
425 µm	7.39	11.85	10.62	5.06	3.78	2.29
300 µm	4.93	8.72	7.21	3.28	2.29	1.69
212 µm	3.55	6.46	5.11	2.88	1.89	1.60
150 µm	2.36	4.80	3.51	2.28	1.39	1.40
106 µm	1.77	5.88	3.61	2.38	1.59	1.50
75 µm	1.08	4.51	2.40	1.69	0.99	1.10
53 µm	0.59	2.15	0.70	0.60	0.30	0.50
Pan	5.02	7.93	4.21	4.57	3.18	4.09
Flow ability metrics[2]						
Angle of Repose,	45.9	48.9	45.7	45.0	44.8	40.5
Compressibility, %	24.7	23.8	23.5	24.1	23.0	20.9
Critical Orifice Diameter, mm	25	27	20	20	22	18
Composite Flow Index	49.6	46.0	56.1	56.1	54.8	63.5
Density, g/mL						
Bulk Density	0.66	0.66	0.68	0.69	0.66	0.72
Tapped Density	0.81	0.87	0.88	0.91	0.86	0.91

[1] The d_{gw} and S_{gw} were determined according to the American Society of Agricultural and Biological Engineers (method S319.4, ASABE, 2008).
[2] Flowability characteristics were measured utilizing methodology outlined by Kalivoda et al., (2017).

Table 4.4 Diet formulation for basal diets across phases

	Starter, d 4-12		Grower 1, d 12-28		Grower 2, d 28-39		Finisher, d39-49	
Ingredient	Corn, %	Sorghum, %	Corn, %	Sorghum, %	Corn, %	Sorghum, %	Corn, %	Sorghum, %
Grain[2]	59.46	59.28	64.98	64.77	69.06	68.81	72.30	72.04
Soybean meal, 46%	34.09	34.28	29.59	29.81	26.24	26.48	23.26	23.51
Limestone	0.90	0.83	1.04	0.96	0.98	0.90	0.97	0.89
Dicalcium phosphate	1.97	2.06	1.02	1.12	0.88	0.98	0.84	0.95
NaCl	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40
L-Lysine HCl	0.17	0.18	0.16	0.17	0.13	0.14	0.12	0.13
DL-Methionine	0.33	0.31	0.29	0.27	0.25	0.24	0.22	0.20
L-Threonine	0.12	0.12	0.08	0.08	0.05	0.05	0.03	0.02
L-Valine	0.04	-	0.02	-	-	-	-	-
Vitamin trace mineral premix[3]	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Phytase[4]	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Choline chloride, 60%	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06
Titanium dioxide	0.50	0.50	0.50	0.50	-	-	-	-
Soybean oil	1.71	1.71	1.61	1.61	1.69	1.69	1.54	1.54
Total	100	100	100	100	100	100	100	100

[1] Diets were formulated to meet or exceed the nutrient recommendations set forth by Cobb Vantress (2022). Crude protein was 22%, 21%, 19%, and 18%, AME was 2,975, 3,041, 3,086, and 3,107 kcal/kg respectively.

[2] Grain was either yellow dent corn or mixed red sorghum locally sourced in Manhattan, KS

[3] NB3000 (NutraBlend, Nevada, MO) provided 40 g zinc, 20 g iron, 40 g manganese, 4500 ppm copper, 600 ppm iodine, 60 ppm selenium, 3.087,000 IU vitamin A, 1,102,500 IU vitamin D_3, 6615 IU vitamin E, 331 mg vitamin K, 441 mg vitamin B_1, 2646 mg vitamin B_2, 11023 mg vitamin B_3, 2646 mg vitamin B_5, 551 mg vitamin B_6, 13 mg vitamin B_7, 276 mg vitamin B_9, 4.41 mg vitamin B_{12}, and 154,323 mg choline per kg of premix.

[4] Quantum Blue 10G (AB Vista, Plantation, FL) 10,000 FTU/g feed additive

Table 4.5 Pelleting temperatures and pellet quality of pelleted diets

	Corn	Sorghum				
Item, %	800	400	600	800	1000	1200
Conditioning Temp., °F						
Starter	181.1	182.8	181.8	180.5	181.4	181.0
Grower I	183.4	180.8	180.9	180.4	181.5	182.5
Grower II	180.7	181.3	181.3	180.9	181.4	181.6
Finisher	181.4	180.8	180.7	181.8	180.5	181.2
Hot Pellet Temp., °F						
Starter	193.5	196.3	193.6	192.9	194.6	193.2
Grower I	188.2	190.9	191.0	189.2	191.6	189.4
Grower II	189.4	192.3	191.6	192.6	193.8	194.2
Finisher	190.6	196.1	194.9	195.4	197.6	196.7
Pellet Durability Index, %[3]						
Starter	67.7	69.6	73.4	70.1	69.0	67.1
Grower I	55.2	38.1	45.9	39.6	38.8	30.9
Grower II	29.0	42.1	24.9	26.9	20.55	28.8
Finisher	27.2	22.2	14.9	14.8	30.7	35.6

[1]Treatments were pelleted using a 1-ton 30-horsepower pellet mill (1012-2 Master Model, California Pellet Mill, Crawfordsville, IN) equipped with an 11/64 in × 1 3/8 in (length: diameter 8) pellet die.
[2]The values for conditioning and hot pellet temperatures are the averages of 3 measurements taken at evenly spaced intervals over the duration of the pellet run.
[3]Pellet durability index was measured using a pellet durability tester (Holeman 100, Tekpro Limited, North Walsham, Norfolk, UK) set to 60 sec in triplicate

Table 4.6 Growth performance of broilers fed diets with corn base or variable particle sizes of sorghum

Item	C800	S400	S600	S800	S1000	S1200	SEM	Lin[2]	Quad[2]
BWG, g									
d 4-12	269	282	276	282	272	279	4.3	0.448	0.480
d 4-28	1,559	1,685	1,596	1,656	1,572	1,636	39.2	0.289	0.229
d 4-39	2,743	3,124*	2,984*	3,020*	2,928	3,153*	69.7	0.991	0.013
d 4-49	3,693	4,067*	4,201*	4,010*	4,065*	4,376*	90.9	0.076	0.056
FI, g									
d 4-12	318	343	328	332	330	338	6.3	0.685	0.085
d 4-28	2,129	2,241	2,195	2,218	2,172	2,211	43.4	0.494	0.469
d 4-39	4,171	4,444†	4,312	4,375	4,347	4,545*	90.5	0.328	0.049
d 4-49	5,871	6,474*	6,600*	6,430*	6,552*	6,880*	138.9	0.059	0.131
FCR, g/g									
d 4-12	1.18	1.23*	1.20	1.21	1.21	1.21	0.011	0.582	0.240
d 4-28	1.37	1.33	1.35	1.36	1.38	1.35	0.014	0.044	0.051
d 4-39	1.49	1.42*	1.43*	1.45†	1.49	1.44*	0.013	0.029	0.059
d 4-49	1.58	1.59	1.57	1.60	1.61	1.57	0.014	0.760	0.144

[1] 864 COBB by product breeders were allotted to 72 pens (n=10/pen) at 4 days of age, to begin dietary treatments of C (corn) or S (sorghum) based diets at variable µm, 400, 600, 800, 1000 or 1200. Body weight gain (BWG), feed intake (FI) data were collected for day 4-12, 13-28, 29-39, and 40-49 with feed conversion ratio (FCR) being calculated for each phase and adjusted.

* Denotes statistical difference ($P \leq 0.05$) between sorghum treatments compared back to the C800 (control) treatment utilizing Dunnet's test.

† Denotes statistical tendency ($0.05 \leq P \leq 0.10$) between sorghum treatments compared back to the C800 (control) treatment utilizing Dunnet's test.

Table 4.7 Gizzard weight relative to body weight and pH in broiler chickens

Parameter	C800	S400	S600	S800	S1000	S1200	SEM	Lin[2]	Quad[2]
BW, g	4,161.4	4,352.3	4,253.1	4,291.8	4,482.6†	4,310.9	105.2	0.63	0.98

Gizzard, g	43.6	37.7*	41.1	44.3	47.7*	43.7	1.41	< 0.01	< 0.01
Relative	1.05	0.87*	0.97†	1.04	1.07	1.02	0.03	< 0.01	< 0.01
Gizzard pH	3.07	3.61*	3.29	3.24	3.11	3.29	0.14	0.05	0.06

[1] 864 COBB by product breeders were allotted to 72 pens (n=10/pen) at 4 days of age, to begin dietary treatments of C (corn) or S (sorghum) based diets at variable microns, 400, 600, 800, 1000 or 1200. On day 49, 2 average birds per pen were sacrificed for the collection of gizzard pH and weight. Gizzard pH was measured using a portable pH and temperature meter (HI99165, Hanna Instruments, Woonsocket, RI). Relative gizzard weight was collected after contents were probed for pH and the organ was rinsed with distilled water. Data are reported as a ratio of gizzard weight to body weight.

[2] Data were analyzed using PROC GLIMMIX of SAS 9.4 with linear and quadratic contrasts

[3] Dunnet's test was utilized for comparison of S treatments back to C (control) treatment.

* Denotes statistical difference ($P \leq 0.05$) between sorghum treatments compared back to the C800 (control) treatment utilizing Dunnet's test.

† Denotes statistical tendency ($0.05 \leq P \leq 0.10$) between sorghum treatments compared back to the C800 (control) treatment utilizing Dunnet's test.

Chapter 5 - Influence of Dietary Protein and Amino Acid Formulation on Intestinal Dynamics, *Clostridium perfringens*, and *Eimeria spp*. in Broiler Chickens: A Review

ABSTRACT

The broiler gastrointestinal tract (**GIT**) hosts a diverse microbiome varying across bird age, diet, and gastrointestinal segment. Although the crop and upper intestine are dominated by *Lactobacillus*, the distal ileum and ceca harbor dense, diverse anaerobic communities that utilize undigested nutrients. Protein digestion is initiated in the proventriculus and gizzard and continues in the SI, where peptide and AA absorption occurs through brush-border transport systems. When AA, peptides, and nitrogenous residues are not digested or absorbed, they become substrates for microbial fermentation, increasing the risk of dysbiosis.

Two major enteric pathogens of economic concern—*Clostridium perfringens* and *Eimeria spp*.—interact with the nutrients and intestinal physiology present in the broiler. *C. perfringens*, an opportunistic Firmicute, proliferates when excess nitrogen and peptides are available or intestinal integrity is compromised. *Eimeria spp*. are intracellular protozoa that damage epithelial tissue, increase mucin turnover, and leak plasma proteins into the lumen, often predisposing birds to necrotic enteritis. Together, these pathogens disrupt nutrient absorption, depress performance, and increase flock mortality.

Dietary protein and AA formulation influence microbial ecology by modifying digestibility, substrate abundance, and intestinal integrity. Precision formulation helps reduce excess nitrogen excretion and limits nutrient availability for pathogenic bacteria.

Protein quality—shaped by AA profile, heat processing, and antinutritional factors—determines digestibility and microbial nitrogen supply. Heat damage can decrease Lys and other AA availability, causing undigested nitrogen to reach the distal ileum and ceca. Integrating protein quality and AA balance into feed formulation provides nutritional leverage to mitigate pathogenic overgrowth and improve broiler GIT health.

INTRODUCTION

The intestinal microbiome is the collective genomes of microorganisms that can be classified as archaea, bacteria, fungi, protozoa, and viruses, which inhabit the gastrointestinal tract (**GIT**) of broiler chicken. Hundreds of microbes interact not only with the host (broiler) but also with other microbes of the microbiome. Although the host GIT is not just made up of microbes, it also contains many different cell types; and within each segment (crop, proventriculus, gizzard, small or large intestine) there are different microenvironments with differing pH levels, functions and nutrient interactions. Each of these microenvironments within the GIT support microbiotas with different classification, functions, and compositions and will vary across age, environment, genetics, and diet.

Microbiota refers to the collective of microorganisms present in a specific environment, while microbial status refers to the quantification of the individual microbes. The microbiota present and microbial status of each microbe of the GIT may directly impact the host animal health, intestinal integrity and ultimately growth performance. Commensal microbes are naturally present in the GIT of the broiler chicken, which work in nutrient digestion, contribute to barrier function, modulate immunity, and stabilize the GIT microbiome. Some commensal microbes may become pathogenic in situations of overgrowth, leading to dysbiosis and poor

growth performance. Beyond commensal microbes there are environmentally introduced microbes that can be introduced into the microbiome.

Among these, *Clostridium perfringens*, a commensal bacterium, and *Eimeria spp*., environmentally acquired protozoa parasite, are two of the most economically significant enteric pathogens in the global broiler industry (Skinner, et al., 2010; Williams, 1999). Individually, each can impair intestinal structure and function, reduce growth performance, and increase mortality through necrotic enteritis (**NE**) and coccidiosis, respectively. When co-occurring, *Eimeria spp*. infection often predisposes birds to *C. perfringens* overgrowth, resulting in synergistically greater intestinal lesions, higher mortality, and larger performance losses. Historically, these diseases have been managed with in-feed antibiotics; however, regulatory restrictions and consumer concerns have accelerated the need for alternative nutritional strategies. The relationship between diet formulation and *C. perfringens* pathogenesis is not fully understood; however, the development of nutritional strategies that reduce the intestinal concentration of *C. perfringens* is critical (Wilkie, et al., 2005). Understanding how dietary protein quality and amino acid (**AA**) balance influence gut microbial ecology may reveal practical means to mitigate these pathogens and reduce associated economic losses. Therefore, the aim of this review is to examine how dietary protein and AA formulation can modulate intestinal dynamics, particularly those related to *C. perfringens* and *Eimeria spp*., in broiler chickens.

PROTIEN AND AA DIGESTIVE PHYSIOLOGY IN THE BROILER

Protein is characterized as organic compounds made up of AA held together by peptide bonds, with ten or more bonded being considered a polypeptide. The chemical composition of a

protein is generally H, C, O and N. The structural components of AA and polypeptides dictate how each fold and bond together to create a specific protein and how it will function. Briefly when protein and AA are ingested into the bird, the beak selects particles and does not grind or reduce particle size of the feed. Feed travels down the esophagus and into the crop. Feed can either enter the crop which is namely for storage and moistening of feed prior to entering the proventriculus or feed can pass through and go straight to the proventriculus. Most digestion and microbial fermentation in the crop are of carbohydrates rather than protein (Abbas Hilmi, et al., 2007). Next in the GIT is the proventriculus or true stomach, it is glandular with a mucosal surface and secretes mucus, produces HCl, and pepsinogen (zymogen form of pepsin), like humans. This is where protein digestion begins. The hormone gastrin initiates the breakdown of proteins in the stomach in the presence of food, leading to the secretion of pepsinogen by the chief cells of the gastric mucosa. Pepsinogen is the inactive form of pepsin, which is activated by the low pH (2-4) in the stomach. When activated, pepsin acts as an endopeptidase which cleaves interior peptide bonds at the carboxyl groups of Trp, Leu, Phe, and Tyr, leaving peptide chains 10-200 peptides long.

Contractions of the proventriculus exchanges digesta to the ventriculus, the next compartment of the GIT. The gizzard is namely the mechanical grinding stomach made up of myelinated muscles and a rough interior surface to promote feed grinding action to reduce particle size and increase surface area for enzymatic hydrolysis. Once broken down finely and mixed well with pepsin, the feed enters the small intestine (**SI**) at the duodenum. Typically, enterocytes in the SI do not have transporters for peptides containing > 4 AA or allow tight junction penetration therefore proteins and polypeptides need to be further digested. When the polypeptides get to the duodenum they are met with several pancreatic peptidases. Three

endopeptidases (cleaving interior peptide bonds)- trypsin, chymotrypsin, and elastase and two exopeptidases (cleaving exterior peptide bonds)- carboxypeptidases A and B enter the SI through the pancreatic duct and are mixed with the digesta, bile and bicarbonate to raise the pH and continue with digestive function. Trypsin cleaves carboxyl side chains of Lys and Arg, chymotrypsin cleaves carboxyl side chains of Phe, Tyr, and Trp, elastase cleaves carboxyl side chains of Ala, Ser, Gly and Val. Carboxypeptidase A cleaves carboxyl side chains of Gly, Ser, Ala, Thr, Tyr, and Trp, while carboxypeptidase B cleaves carboxyl side chains of His, Arg, and Lys. The polypeptides that had entered the duodenum are hydrolyzed and broken down to single AA, dipeptides, and tripeptides making their way to the jejunum and ileum. The jejunum and ileum are lined with microvilli, secreting enzymes (aminopeptidase N, aminopeptidase A, dipeptidyl peptidase IV, and carboxypeptidase) to further break down the AA, dipeptides, and tripeptides. Aminopeptidase N cleaves at the N terminal of Arg, Leu, and Met, whereas aminopeptidase A cleaves at the N terminal of Arg, Lys, Glu, and Asp. Dipeptidyl peptidase IV cleaves Pro and Ala at the N terminal. Carboxypeptidase on the other hand cleaves at the carboxyl end and of most AA. The brush borders in the jejunum and ileum facilitate absorption through the active transport of proteins into the bloodstream. PEPT1 makes up about 40% of the transportation at the brush border and uses secondary active transport via H ions. It is best for L-AA isomers and ions with a charge less than 2+. A Na ion gradient occurs at the basolateral membrane via Na/K pumps which allows the Na/H pump to push out H as it draws in Na. This allows H to cotransport dipeptides and tripeptides through PEPT1 transporters. Amino acid transporters make up the other 60% of the brush boarder transportation. Different AA transporters are based on the type of AA base structure and charge. At the brush border, these transporters use active transport via Na ions and AA exchange to bring AA into the enterocyte.

Multiple transporters transport multiple AA and can become competitive in nature. B0 and ASC transport neutral AA like Ala, Tyr, Ser, Thr and Cys. B0 also transports cationic AA, Lys, His, and Arg. X-AG and ASC transport anionic AA, Glu, and Asp. Imino, and B0 transport Pro and Gly. The primary digestion and absorption are conducted in the duodenum and jejunum while the ileum is the clean-up section where it catches nutrients that were not absorbed prior. Although the ileum does not have a high capacity for absorption due to reduced receptors and enzymatic activity. Undigested and unabsorbed nutrients that make it to the distal SI and ceca may go to aid microbials via energy and fuel for function. Although most proteins are broken down into single AA, dipeptides, and tripeptides to be transported into the enterocytes in the SI, some whole proteins, can be passively diffused or paracellularly enter through tight junctions.

When AA, dipeptides, tripeptides, non-protein nitrogen (**NPN**) and undigested proteins are not absorbed, they become a nutrient source for the microbes present in the SI and large intestine (**LI**) which may lead to dysbiosis. Minimal benefits for the host exist when unabsorbed N, AA, or peptides reach the microbial populations. The ceca come after the ileo-cecal-junction which transitions the SI to the LI. These are a pair of blind sacs which carry a large capacity for anerobic fermentation for nitrogenous substrates. This fermentation can produce short chained fatty acids (**SCFA**), branched chain fatty acids and toxins. After fermentation in the ceca the colon (true LI) handles water, electrolyte, and SCFA reabsorption with the remaining unabsorbed materials being excreted as waste.

MICROBIAL ECOLOGY

Although archaea, bacteria, fungi, protozoa, and viruses are all present in the GIT, they are present in varying amounts. On a kingdom level, bacteria are greater than 99% of the

microbiota (Clavijo and Flórez, 2018) and not all are commensal. Archaea and fungi account for less than 1% each of the biomass of the microbiota (Davies, et al., 2022; Saengkerdsub, et al., 2007). Whereas viruses and protozoa are not detected or measured with typical 16S RNA sequencing. Viruses may contribute more particle numbers but account for little biomass in DNA-based analysis (Sausset, et al., 2020). Limited data exists around commensal protozoa community in the GIT of broilers but commonly protozoa like *Eimeria spp*., are introduced and inhabit the GIT. In the egg, the chick's microbiota composition is relatively naïve with low densities of microbes but quickly begins to diversify and populate with exposure outside of the egg (Ding, et al., 2017; Stanley, et al., 2014). The microbiota in the first few day's post-hatch is usually dominated by *Enterobacteriaceae*, the microbiota increases in complexity and there is a shift from Gram-negative to Gram-positive bacteria, and *Firmicutes* and *Bacteroidetes* start to dominate with the microbiota composition continuing to develop attaining a mature configuration by week two or three (Ranjitkar, et al., 2016). *Firmicutes* are a major phylum of gram-positive bacteria in the GIT of the broiler, which includes the classes of *Bacilli* (lactic acid producing and spore forming) and *Clostridia* (obligate anaerobes producing SCFA). *Proteobacteria* is another common phylum found in the broiler GIT which are gram negative rods, facultative anaerobes, and known to ferment glucose and reduce nitrate. These microbes can be considered opportunistic or may become pathogenic (e. g., *Escherichia coli, Enterobacter spp., Salmonella spp.,* and *Pathogenic E. coli*). Another common phylum found in the broiler GIT is *Actinobacteria* (e.g., *Bifidobacterium*) which are gram-positive, non-spore forming, obligate anaerobes which ferment carbohydrates and produce acetate and lactate (Wei, et al., 2013).

Populations of microbes differ within each compartment of the GIT over time with diversity and populations dependent on environmental exposure, diet, age, and genetics. The crop although considered a storage area for feed also contains a microbial community for fermentation dominated by *Lactobacillus* species, and some *Clostridium, Bifidobacterium, Enterobacteriaceae,* and *Enterococcus* (Oakley, et al., 2014; Rehman, et al., 2007). Some research is indicative that starch hydrolysis and partial protein hydrolysis begins in the crop with glucose being absorbed and being fermented by the crop microbial population producing lactic acid and SCFA (Svihus, 2014). However, the crop's ecological environment is more dependent on fiber level (Bayer, et al., 1978) or feed restriction (Hinton, et al., 2000), rather than protein. The crop is considered one of the main sites of bacterial activity, with the caeca being greater than the crop and to a similar or lesser extent, the small intestine (Gabriel, et al., 2006).

The proventriculus is less explored when considering microbial ecology, due to its glandular and acidic nature but much of its microbial community is *Lactobacilli* (Oakley, et al., 2014). Although a shared compartment with the proventriculus, the gizzard shares a similar microbial community to that of the crop (Sekelja, et al., 2012) but all three areas of the GIT are relatively dominated early on by *Lactobacilli* due to the nature of the acidic environment (pH 2-5). *Lactobacilli* allow carbohydrate fermentation, limiting energy sources for slower-growing, pathogenic species further on in the GIT. *Lactobacilli* produce lactic acid, acetic acid, and occasionally reuterin--a broad-spectrum antimicrobial compound produced by *L. reuteri* that attacks gram-negative and gram-positive bacteria (Talarico and Dobrogosz, 1989). These may contribute to the inhibition of prevalent pathogens like *Salmonella, C. Perfringens* and *E. coli*.

When considering the microbial communities of the small intestine (SI), the duodenum, jejunum, and ileum can each be viewed as distinct microbial microenvironments. However,

across these segments, the dominant genera are typically *Lactobacillus*, followed by members of *Clostridiaceae, Streptococcus*, and *Enterococcus* (Lu, et al., 2003). In the first two weeks of development of the chick GIT, the duodenum exhibits relatively greater microbial diversity, but by the end of the broiler production cycle (approximately d 42–49), the duodenal community becomes overwhelmingly dominated by *Lactobacillus* species (~99%) (Han, et al., 2016), reflecting the strong selective pressure for acid-tolerant, rapidly fermenting taxa.

The jejunum and ileum similarly contain communities enriched in *Firmicutes*, which generally comprise more than 60% of microbial composition throughout the SI (Xiao, et al., 2017). These regions increasingly shift from lactate-producing to mixed fermentative populations as digesta moves distally, and although still less diverse than the ceca, the ileum is markedly more diverse than the proximal SI. The ileum also represents a key interface between digestion and fermentation, where undigested proteins and carbohydrates enter fermentation pathways and influence microbrial community structure.

In contrast, the ceca represent the most microbially dense and diverse regions of the broiler GIT (Stamilla, et al., 2021). While *Firmicutes* remain abundant, the taxonomic profile shifts substantially, the Bacilli-enriched community characteristic of the proximal SI transitions to dominance of Clostridia (e.g., *Clostridium, Ruminococcus, Faecalibacterium*) and Bacteroidetes (e.g., *Bacteroides*), reflecting the specialized anaerobic fermentation (Brisbin, et al., 2008). The ceca support extensive fermentation of otherwise indigestible substrates, generating SCFA and other metabolites that modulate nutrient utilization, gut barrier function, and immune responses. The colon has a similar community to that of the ceca however carries a lower microbial population by mass. There is a mixture of anerobic and facultative microbes like the transient ileal communities *Clostridia*, *Lactobacillus*, *Enterococcus* and some

Proteobacteria, but the shorter retention time and different substrate flux results in a community that is less specialized for fermentation (Oakley, 2016). The colon acts as a water and electrolyte reabsorption site and salvages metabolite products from the microbes and plays a role in N cycling when proteolytic fermentation has occurred.

Although each compartment of the GIT of the broiler carries a slightly unique microbial community, the GIT carries' similar profiles throughout. As discussed earlier, when proteins, peptides, AA and NPN reach the distal SI and LI, the microbes will utilize the nutrients as fuel for their homeostasis and growth. Some microbes exemplify how ecology, diet, and intestinal integrity work on an axis of balance and when the balance starts to tip one way or another, opportunistic microbes can become pathogens. *C. perfringens* is one of those opportunistic, proteolytic *Firmicutes* that blooms under protein-rich, dysbiosis conditions and commonly in concert with *Eimeria spp*., an intracellular protozoa that cause mucosal damage, creating nutrient-rich niches that participate in secondary bacterial overgrowth.

Clostridium perfringens. *C. perfringens* is a commensal, Gram-positive, spore-forming, obligate anaerobe belonging to the phylum Firmicutes. The organism is taxonomically divided into seven toxin types (A–G) based on its extracellular toxin profile, collectively capable of producing six different toxin types. Among these, types A, C, and more recently G are most associated with clinical NE in poultry due to their expression of toxins including alpha, beta, and NetB toxins (Immerseel, et al., 2004; Rood, et al., 2018).

Outside of the host, *C. perfringens* persists in litter, dust, soil, and fecal material as environmentally resilient heat-resistant spores, which facilitate transmission and allow for rapid overgrowth when intestinal or environmental conditions become permissive (Li, et al., 2016). In

healthy broiler chickens, *C. perfringens* is typically present at low abundance (~10^4 CFU/g digesta) within the SI and ceca (Barnes, et al., 1972; Kondo, 1988; Timms, 1968). However, clinical overgrowth is generally characterized by luminal concentrations of approximately 10^6–10^9 CFU/g digesta (McDevitt, et al., 2006; Williams, 2005), at which point toxin production and lesion development become disruptive. *C. perfringens* can be quantified through either culture methodology or real time PCR 16S rDNA sequencing (Johnson, et al., 2019; Wu, et al., 2011). These toxins contribute to mucosal necrosis—primarily in the small intestine—leading to dysbiosis, reduced feed intake, impaired nutrient absorption, and potentially severe mortality (Kulkarni, et al., 2020).

Predisposing factors of a bloom in *C. perfringens* in the intestine could be increased pH, decrease passage rate, preexisting dysbiosis from stress, increased N, AA or peptide substrate availability, changes in microbial populations, or coinfection causing epithelial damage (Moore, 2016). Interaction of *C. perfringens* with *Eimeria spp*., is an important factor in the pathogenesis of NE, commonly, the gut epithelial damage associated with NE coincides with an infection of coccidiosis with genus *Eimeria*, often linking as one set of similar symptoms (Williams, 2005). In short, *Eimeria* parasites induce leakage of plasma proteins by adhering and lysing epithelial cells as a consequence of the intracellular stages of their lifecycle. According to (Collier, et al., 2008), a cocci infection enhances mucus production through goblet cell activation through damaging the epithelium in the intestine. Both effects provide an increase in available nutrients to microbes and create an environment favorable for proliferation of *C. perfringens*.

Eimeria spp. *Eimeria spp*. are obligate intracellular protozoa (phylum Apicomplexa) that infect intestinal epithelial cells of broilers and are responsible for coccidiosis. Following ingestion of

sporulated oocysts, sporozoites excyst and invade intestinal epithelial cells, undergoing sequential asexual and sexual replication before new oocysts are shed into the environment, where sporulation occurs under warm, humid conditions. Although not commensal, there are seven (*E. acervulina, E. brunetti, E. maxima, E. mitis, E. necatrix, E. praecox and E. tenella*) common species found in broilers in the United States that are linked to coccidiosis development. Species differ in disease pathogenicity, infection cite and clinical signs/lesion severity (Kimminau, 2015). Pathogenicity is limited to sporulated oocyst count with several studies suggesting infection behaves in a dose dependent manner and that typically more than one species is present (Williams, 1973; Williams, 2001). *C. perfringens* can be measured as CFU/g of digesta, but *Eimeria spp.* is measured as oocyst per gram of digesta or excreta. No baseline or infectious level has been established due to oocyst count being reliant on sampling method, vaccination status and program, age of the bird and species of *Eimeria.* Oocyst per gram of digesta or excreta should be correlated back to symptoms like intestinal lesion scores, dysbiosis, or decreases in feed conversion.

E. acervulina develop in the duodenum, *E. maxima, E. mitis, and E. necatrix* target the jejunum extending to the ileum, *E. tenella* primarily colonizes in the ceca (Long and Joyner, 1984) and *E. brunetti* is typically found in the lower LI and rectum. Infection results in enterocyte destruction, villus atrophy, crypt hyperplasia, hemorrhage, increased mucus production, and disruption of tight junctions, leading to decreased digestion and absorption of nutrients, reduced enzyme activity, immune activation, and diminished growth performance (Chapman, 2014; Williams, 2005). Subclinical infection is commonly characterized by mild lesions, impaired feed efficiency, and increased susceptibility to secondary pathogens like *C. perfringens* via plasma protein leakage and decreased digestion and absorption (Elwinger, et al.,

1992; Peek and Landman, 2011). Eimeria attack the two major barrier components in the GIT; the mucosal layer responsible for protecting the enterocytes and then the enterocyte layer (epithelium) which encompasses the protecting the rest of the body from intestinal contents.

Coccidiosis control is primarily achieved through anticoccidial drugs (ionophores, chemical coccidiostats) and live, attenuated, or non-attenuated vaccines, each of which influences intestinal microbial ecology and nutrient dynamics (Ruff, 1999). Vaccination induces controlled cycling of *Eimeria*, causing mild epithelial damage and increased mucin turnover, which may elevate AA requirements and increase substrates for opportunistic pathogens such as *C. perfringens* (Collier, et al., 2008). These mitigation strategies may modify the degree of mucosal injury, immune activation, and nutrient escape into the hindgut, ultimately impacting the microbiota and intestinal integrity. The synergistic relationship between *Eimeria*-induced mucosal injury and *C. perfringens* overgrowth highlights the interconnectedness of parasitic, microbial, and nutritional factors in GIT health and integrity, emphasizing the importance of integrated control strategies including dietary formulation, microbial modulation, and prevention (Kers, et al., 2018).

PROTEIN CONSIDERATIONS IN FEED FORMULATION

Protein in formulation. Protein is a critical macronutrient in broiler feed formulation, providing the AA necessary for rapid growth and metabolic function. However, beyond its nutritional role, the quality and digestibility of dietary protein strongly influence intestinal microbial ecology and the prevalence of enteric pathogens such as *C. perfringens* and *Eimeria spp*. In practice, nutritionists design diets to meet the bird's AA requirements rather than total CP. This approach ensures that nutrient supply aligns with the animal's genetic potential and minimizes excess N

excretion that can fuel pathogenic bacteria in the distal ileum and ceca. Essential AA (**EAA**; those not synthesized in sufficient quantities endogenously) are prioritized in formulation, while nonessential AA (**NEAA**; synthesized in sufficient demand for maintenance) are often supplied indirectly through protein-bound sources or metabolic pathways.

Modern formulations have removed crude CP minimums, and crystalline AA maximums (Kidd, et al., 2021), often including supplementation of up to seven key essential AA: Lys, Met, Thr, Val, Ile, Trp, and Arg. This is due to the ideal protein concept. Methionine and TSAA (Cys + Met) are generally consider the first limiting AA in poultry with consideration that Lys may become limiting dependent on diet, and production goal of the animal.

Amino acids are commonly formulated on a digestible basis, using apparent or standardized ileal digestibility (**AID, SID**) values. Ileal digestibility refers to the disappearance of AA that were in the diet and measured from consumption to the distal ileum. Apparent ileal digestibility refers to the calculation of total ileal AA outflow from AA ingested in the diet, whereas SID is calculated utilizing AID and is corrected for ileal endogenous losses **(IAA**). Some SID values may be corrected using book published values of IAA or when conducting specific experiments, researchers can measure these by fasting a block of broilers in the research study (Engster, et al., 1985). Using SID values allows nutritionists to accurately compare ingredients and improve formulation precision—reducing the risk of over-formulation, nitrogen excretion, and undigested protein residues that promote *C. perfringens* proliferation and *Eimeria spp*. severity. Other considerations when formulating protein into a broiler diet go beyond least cost formulation.

Nutritionists should consider nutrient variation within feed ingredients, as processing batch, plant of origin, and annual crop conditions can influence nutrient composition and

digestibility. Although soybean meal (**SBM**) is considered relatively consistent, SID Lys and other AA show between batch variation driven by origin and heat treatment (Hemetsberger, et al., 2021). Canola meal (**CM**) exhibits greater variability in SID AA, reflecting differences in oil extraction and fiber content (Adewole, et al., 2017). Fermented products such as corn distillers dried grains with solubles (**cDDGS**) are among the most variable, where variable drying temperatures and solubles addition can depress SID Lys too, often with >15–20 percentage fluctuation (Wang, et al., 2020). These differences highlight the importance of ingredient characterization and continuous monitoring of nutrient profiles. Bird genetics and age, ingredient quality, AA profile and digestibility of the protein source, antinutritional factors, and health management should be considered in feed formulation. Ultimately the goal is to minimize unabsorbed nitrogen in feed formulation due to cost, animal health and environmental concerns.

Protein quality. There are several things to consider when referring to protein quality and some parameters may be more important to specific protein sources than others. Nutritionists evaluate protein characteristics like AA profile, digestibility, antinutritional factors, nutritional variation, and processing damage. Protein quality is most importantly based on AA profile and the digestibility of the AA within the feed. Quality parameters can be measured utilizing *in vitro* or *in vivo* methods. *In vivo* digestibility studies are limited by animal utilization, money and turnaround of results but are the only way to determine digestibility of feed ingredients. Therefore, extensive research has been conducted to connect feed quality parameters back to AA digestibility of feed ingredients (Aderibigbe, et al., 2020; Frikha, et al., 2012; Parsons, et al., 1991). For more rapid analysis, nutritionists rely on *in vitro* chemical analysis like AA concentration of feed ingredients or complete feed, urease activity, protein dispersibility, reactive Lys, protein solubility with potassium hydroxide (**KOH**), and trypsin inhibitors (**TI**). Heat

treatment is utilized as a pathogen kill step, reduces antinutritional compounds, and helps to complete the finished product into a usable form in feed manufacturing.

Urease is typically a concern in SBM, CM, and legume seeds. Urease activity testing could be used as an indirect measure of TI activity, based on the assumption that the heat denatures TI and urease to a similar extent. Commonly heat-processed products can be undercooked or overcooked which lowers the protein and AA digestibility of a feed ingredient. Undercooking may result in the incomplete destruction of antinutritional factors and is routinely measured by the urease activity test. Overcooking may increase Maillard reactions, which reduces Lys and other AA availability, commonly assessed by the KOH protein solubility test (Ravindran, et al., 2014). In brief, the Maillard reaction is the formation of pigmentation when a reducing sugar condenses with the amino group of an AA. There are seven reactions across three stages. In SBM, Lys is commonly the most susceptible due to its side chain. The Lys in an ingredient that underwent the Maillard reaction is still present in total AA concentration analysis but is not available to the broiler; leaving excess N undigested and headed to fuel microbes.

Specifically, urease activity is measuring the change in pH of a sample. When urease is present, urea is hydrolyzed producing CO_2 and ammonia, which increases pH. Urease is heat instable similar to TI and therefore when SBM or other ingredients are cooked appropriately there are negligible amounts. However, this should not be relied on as a sole measure because urease activity may be low, but overcooking may have occurred. A baseline level of TI of <4 mg/g TI is recommended (Clarke and Wiseman, 2005). Nutritionist will pair urease activity with KOH or reactive Lys assays to better understand overprocessing characteristics and nutrient availability (Huisman, 1990).

Decreased KOH and reactive Lys have been linked to decreased digestibility of AA, specifically Lys, due to the Maillard reaction (Caprita, et al., 2010). Some of the methods used to determine the concentration of reactive Lys in heat-damaged proteins are the homoarginine procedure, the furosine procedure, and the fluorodinitrobenzene procedure (Oliveira, et al., 2021), all of which are indirect methods of measuring reactive Lys but can be more indicative of availability to the broiler than just total AA concentration of an ingredient or feed. The homoarginine procedure transforms the unbound Lys to homoarginine through a guanidination reaction inducing a color change which can be quantified. Furosine is a product of the Amadori compounds in the Maillard reaction. Therefore, it can be used to measure the unreactive Lys and subtracted from the analyzed total Lys concentration to calculate the available Lys. Fluorodinitrobenzene binds reactive Lys and will change color and therefore can be quantified with a spectrometer. This method can have interference when carbohydrates are present. Research comparing KOH to other measures of protein quality indicate that KOH solubilities between 78-84% are optimal for animal performance. While values over 84% are under processed and values under 74% are over processed and will have reduced Lys digestibility (Dozier, et al., 2011). Another test that could be paired with urease activity or TI is the protein dispersibility index. Similar to KOH solubility, utilizing water instead of KOH, protein dispersibility of 40-45% indicate neither over nor under processing has occurred (Batal, et al., 2000).

Most protein sources utilized in broiler diets are by-products of other industries like human food or ethanol and can be generally classified under plant or animal origins. These protein sources may undergo further processing or heat treatment prior to becoming a finished feed ingredient. How each protein source is processed may impact the AA profile subsequently

effecting overall protein quality and value to the broiler as described above. Common plant-based proteins used in broiler diets include SBM, CM, and cDDGS. Animal-based proteins, including meat and bone meal (**MBM**), feather meal (**FEM**), and fishmeal (**FM**), are also used depending on the economics of utilization and availability. Plant and animal protein ingredients vary widely in digestible AA content (Table 1) anti-nutritional factors, and heat damage, which all can influence digestibility and ultimately the extent of AA delivery to the host versus the microbes (Barua, et al., 2020). Generally nutrient availability and quality parameters should be continuously monitored due to nutritional and antinutritional variations within protein source, heavily dependent on origin, processing batch and plant.

AA Profile and Digestibility on C. perfringens and Eimeria spp. Key quality factors when considering a protein source is the AA profile and how digestible or available those proteins and AA are for the broiler to utilize. For broilers, nutritionists are looking for protein sources higher in digestible Lys, and Met or TSAA, to promote growth and maintenance and then will supplement with crystalline AA to achieve minimal CP inclusion and N excretion. Genetic suppliers have given baseline AA requirements for the modern broilers refined by external research at universities and commercial field trials. The overarching goal is aligned with the ideal protein concept aiming to provide EAA in appropriate proportion to one another without deficiency or excess, thereby maximizing utilization while minimizing AA overflow to the distal intestine where microbial fermentation occurs (Emmert and Baker, 1997). Although even with the highest quality diet, not all dietary protein is captured by the host and some protein will be endogenous losses even under uninfected conditions.

Undigested AA and peptides entering the distal ileum and ceca provide substrates for proteolytic fermentation. This activity alters microbial community structure and increases the growth potential of proteolytic pathogens such as *C. perfringens*. Excess AA compete for transport receptors in the SI, decreasing absorption efficiency and further increasing substrate flow to the distal SI and ceca. When AA and peptides reach the microbes, in contrast to carbohydrate fermentation—which yields short-chain fatty acids (SCFA)—proteolytic fermentation, or putrefaction, produces metabolites including ammonia, biogenic amines, phenols, indoles, hydrogen sulfide, and branched-chain fatty acids, several of which are cytotoxic, pro-inflammatory, or alkalinizing to the lumen (Apajalahti and Vienola, 2016). These products can become toxic to the GIT and increase luminal pH, decreases colonization resistance, damage the epithelium, and promote proteolytic bacterial overgrowth, including *C. perfringens*, potentially leading to NE (Adil and Magray, 2012; Broom, 2017).

To minimize substrate availability to microbes, some have attempted to manage *C. perfringens* infection with the reduction of CP in the diet (Drew, et al., 2004). However, when *Eimeria spp*., are present, reducing CP has minimal effects due to the lysing of cells and their contents flowing downstream in the lumen, still providing nutrient substrate to *C. perfringens* (Hilliar, et al., 2020). Several have reported that reducing CP and utilizing crystalline AA to balance requirements does not hinder growth performance (Belloir, et al., 2017; Bregendahl, et al., 2002; Laudadio, et al., 2012). Some have reported an increased risk of NE with the utilization of animal protein ingredients such as FM or MBM (Kulkarni, et al., 2020; (Shojadoost, et al., 2012). Drew et al., (2004) reported that reducing CP level of either FM or SBM reduce *C. perfringens*, noting that FM has a particularly higher counts of *C. perfringens* in the ileum and ceca, compared to the SBM treatments which aligns with challenge researchers using FM to

exacerbate NE challenges (Truscott and Al-Sheikhly, 1977). The increased *C. perfringens* could be attributed to the increased Met and Gly level in FM compared to SBM. Wilkie et al., (2005) implicates Gly when they observed a positive correlation of Gly in digesta to *C. perfringens* CFU/g of digesta. This supports Gly as a required AA to produce α-toxin in media (Stevens and Rood, 2006). Although Met was suggested to be hindering in Drew et al., (2004), others have observed low CP diets supplemented with high Met (3-4x requirement) hindered the growth of *C. perfringens* (Dahiya, et al., 2007). Collectively data suggests nutritionist should monitor specific excess AA or more specifically Gly in the diet to ensure minimal substrate reaches *C. perfringens* (Xue, et al., 2017).

Arginine is known for its role in intestinal integrity, but under NE challenge, Arg and Pro metabolism pathways are enriched (Zhang, et al., 2018) and can serve as an energy source for pathogens. The microbes may degrade Arg, producing urea and ammonia, diminishing available Arg for the broiler. Zhang et al., (2018) observed that accelerated Arg metabolism by the microbiota in challenged birds may cause deprivation in the host, leading to compromised T-Cell function and eventually resulting in increased susceptibility to infection (Popovic, et al., 2007). Methionine and TSAA requirements have not been found to be different when broilers are under *C. perfringens* or *Eimeria spp.* challenges (Ren, et al., 2020; Rochell, et al., 2016). Although, *Eimeria spp.* infections have been known to significantly decrease AA digestibility due to intestinal damage (Adedokun, et al., 2016). Although not directly impacting the bloom of *C. perfringens*, indirectly Thr, Gln, and Arg have been shown to help intestinal integrity through modulation of mucin synthesis and barrier integrity (Ebadiasl, 2011; Moghaddam, et al., 2011; Murakami, et al., 2007; Tan, et al., 2014) which may help reestablish intestinal integrity post infection. It is suggested that during times of impaired absorbative capacity, highly digestible

AA can restore some lost growth performance and contribute to rebuilding impaired intestinal integrity (Bortoluzzi, et al., 2018). Reducing dietary CP may not reduce the effects of NE, especially when coccidiosis is the predisposing factor but increased concentrations of specific AA in broiler diets may help support GIT recovery and hinder *C. perfringens* bloom.

Reducing dietary CP while meeting SID AA requirements via crystalline AA can lower nitrogen excretion and reduce distal gut substrate for proteolytic fermentation, through ingredient digestibility and practical diet formulation (Macelline, et al., 2021; Macelline, et al., 2020). Measures such as SID AA supply, reactive Lys, KOH protein solubility, and other heat-damage indicators could be more informative indicators of functional nitrogen quality than CP alone and represent important parameters that should be incorporated into microbial risk models for NE (Kim, et al., 2012; Sharareh, 2021). Total nitrogen flow to include the consideration of IEAA losses is not used in practice. Although quantification of these losses in unchallenged and challenged models would be helpful to improve the efficiency of protein and AA formulation when faced with enteric challenges.

Protein source matters as specific animal proteins and heat-damaged plant proteins are repeatedly implicated in NE challenge models implicating poor digestibility (Dahiya, et al., 2006). Thus, the AA profile alone is insufficient; digestibility of the AA profile directly determines how much protein ultimately supports growth versus hindgut microbial proliferation. Ultimately in consideration of the commonly used protein sources in broiler feed, origin and processing of ingredients heavily influences digestibility and downstream microbial nitrogen availability. Collectively, these factors determine whether dietary protein supports bird growth or inadvertently feeds pathogens.

APPLICATIONS AND IMPLICATIONS

Microbes play a key role in the GIT function and homeostasis. Feed formulation can directly impact the status of GIT health and the microbiota. Herein it has been discussed how microbial ecology works within the GIT and how protein and AA in feed formulation directly impact pathogenesis of *C. perfringens* and *Eimeria spp.* Industry has moved to lower CP diets and an increase in utilization of crystalline AA. However, nutritionists still heavily rely on heat processed protein sources from plants and animals for the larger portions of the broiler diet. Therefore, special attention should be made to protein quality—AA profile, digestibility, antinutritional factors and chemical characteristics—when formulating a diet for at risk broilers. Reducing CP alone is not as beneficial as considering the origin of the protein source, where plant proteins are more preferred in mitigation of *C. perfringens* bloom due to their reduced Gly concentration.

Although industry has moved to more of a nitrogen consciousness in feed formulation by utilizing SID values to formulate with, it could be taken a step further by observing actual total nitrogen instead of CP as our basis. Crude protein has long been included in proximate analysis but often under or overestimates nitrogen of protein due to fixed abundance in calculations. Taking formulation to considering an actual total nitrogen versus CP estimate would require more frequent AA concentration analysis. Laboratory analysis can be costly but technology like near-infrared spectroscopy may enable a shift to total protein nitrogen consideration in feed formulation.

Despite advances in the understanding of how broiler nutrition impacts enteric pathogen management, basic knowledge gaps remain regarding the mechanisms among dietary protein formulation, and intestinal microbes, *C. perfringens*, and *Eimeria spp.* A part of the challenge is

that there is no single standard gut microbiota composition shared by broiler chickens. Another part of the challenge is that other factors such as carbohydrates, fats and feed additives impact microbial ecology too. Future work should prioritize defining nitrogen and AA flow along the GIT under commercial feeding conditions and observing other nutritional factors. Although protein AA profile and digestibility are recognized as the general classifications of protein quality related to NE, the specific contributions of protein source, processing conditions, and antinutritional factors still needs further investigating, including assessment related back to *in vitro* parameters of protein quality.

Beyond nutrient effects, toxin expression in *C. perfringens* is regulated through peptide-sensing two-component systems indicating that luminal peptides may serve as signaling cues regulating virulence gene expression, including NetB. This suggest that dietary AA affect both host mucosal physiology and microbial virulence signaling, yet mechanistic integration of these interactions remains limited and warrants further investigation. Indicating research is needed on individual AA and how they modulate pathogenesis under *Eimeria* infection and *C. perfringens* challenge. Improved understanding of how AA regulate virulence of toxins could identify nutritional strategies for suppressing specific toxin production. A deep integration of metabolomics, proteomics, and transcriptomics is needed to provide further insight into protein–pathogen signaling pathways and impact on toxin production. Collectively, future research must take an integrated approach combining nutrition, feed quality, microbiology, and immunology, to develop robust dietary strategies to mitigate NE and coccidiosis while maximizing broiler performance.

CONCLUSION

Protein quality and AA balance strongly influence microbial ecology, intestinal integrity, and susceptibility to *C. perfringens* and *Eimeria* associated disease. Excess or poorly digested protein increases nitrogenous substrates for pathogen growth, while mucosal injury from coccidia cycling further predisposes birds to dysbiosis. Precision formulation using digestible AA values and rigorous ingredient quality evaluation reduces substrate availability to the microbes. Implementation of formulation with consideration of actual nitrogen values of protein may be more effective than formulating with SID values.

These findings reveal that optimizing protein quality, AA balance, and digestibility is critical considerations to limiting nitrogen flow into the lower intestine, because undigested AA and peptides potentiate *C. perfringens* growth and pathogenesis. Optimizing protein in feed formulation with coinfection of *Eimeria* also helps to recover the intestinal integrity from mucosal leakage and protein flow. Ultimately, precision protein and AA feed formulation represents a practical means to break the synergistic cycle between *Eimeria*-driven mucosal damage and *C. perfringens* overgrowth and toxinogenesis, strengthening intestinal integrity and bird performance in non-antibiotic commercial systems.

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Table 5.1 AA concentration and SID value of common protein sources used in broiler feed formulation

	SBM[2]		Corn DDGS[3]		Canola Meal[4]		Fish Meal[5]		Feather Meal[6]		Meat &Bone Meal[7]	
%	Concentration	SID[8]	Concentration	SID	Concentration	SID	Concentration	SID	Concentration	SID	Concentration	SID
CP	46.6	42.3	27.5	-	36.2	27.3	54.6	47.4	76.2	54.5	47.4	38.1
Lys	2.87	2.60	0.84	0.50	2.02	1.63	3.33	2.89	2.41	1.73	2.44	2.03
Met	0.62	0.57	0.55	0.48	0.78	0.70	1.229	1.15	0.73	0.57	0.62	0.50
Thr	1.82	1.57	1.00	0.77	1.57	1.26	2.20	1.86	3.42	2.34	1.53	1.23
Trp	0.66	0.59	0.21	N/A	0.46	0.39	0.44	0.38	0.62	0.46	0.24	0.19
Arg	3.43	3.18	1.20	1.07	2.25	2.03	3.25	2.90	5.07	4.04	3.51	2.93
Val	2.27	1.99	1.37	1.05	1.67	1.38	2.59	2.22	5.27	3.96	1.98	1.60
Ile	2.18	1.93	1.04	0.87	1.31	1.09	2.08	1.91	3.22	2.58	1.29	1.08
Leu	3.59	3.20	3.20	2.91	2.53	2.20	3.52	3.06	6.20	4.81	2.60	2.15
His	1.23	1.09	0.72	0.63	1.00	0.88	1.03	0.85	1.10	0.78	0.78	0.63
Phe	2.39	2.16	1.35	1.19	1.41	1.23	2.05	1.79	3.54	2.84	1.32	1.07
Ala	2.08	1.73	1.96	1.69	1.55	1.31	3.51	3.12	3.72	2.98	3.82	3.33
Cys	0.68	0.58	0.50	0.42	0.97	0.72	0.96	0.77	3.25	1.79	0.36	0.29
Tyr	1.70	1.50	1.09	0.97	0.93	0.78	1.53	1.21	2.15	1.59	0.94	0.76
Gly	2.02	1.66	1.10	N/A	1.69	1.47	5.01	4.19	6.13	4.80	7.45	6.28
Ser	2.52	2.25	1.27	1.07	1.50	1.24	2.67	2.41	7.26	6.01	1.85	1.50
Pro	2.37	2.11	2.09	1.02	1.95	1.60	3.87	3.42	7.26	6.46	4.19	3.45
Glu	4.55	4.20	4.53	3.94	2.37	2.15	3.74	3.35	5.03	4.51	3.19	2.80
Asp	3.30	2.93	1.82	1.40	1.44	1.31	2.58	2.14	3.23	2.31	1.90	1.33

[1] Adapted from Brazilian Tables for Poultry and Swine, Tables of chemical composition and digestibility on as fed basis; and Hans H. Stein Monogastric Nutrition Group Ingredient Database.
[2] Soybean meal, 46% CP (Rostagno, et al., 2017)
[3] Corn dried distillers' grains with solubles, 6-9% oil (Hans Stein Monograstric Nutrition, 2014); N/A-not reported
[4] Canola meal (Rostagno, Albino, Hannas, Donzele, Sakomura, Perazzo, Saraiva, Teixeira de Abreu, Rodrigues and de Oliveira, 2017)
[5] Fish meal 54% CP, (Rostagno, Albino, Hannas, Donzele, Sakomura, Perazzo, Saraiva, Teixeira de Abreu, Rodrigues and de Oliveira, 2017)
[6] Feather meal, 75% CP, (Rostagno, Albino, Hannas, Donzele, Sakomura, Perazzo, Saraiva, Teixeira de Abreu, Rodrigues and de Oliveira, 2017)
[7] Meat and bone meal, 45-50% CP, (Rostagno, Albino, Hannas, Donzele, Sakomura, Perazzo, Saraiva, Teixeira de Abreu, Rodrigues and de Oliveira, 2017)
[8] SID of AA; Concentration multiplied by SID coefficient