Reducing protein and supplementing crystalline amino acids, to alter dietary amino acid profiles in birds challenged for subclinical necrotic enteritis

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ABSTRACT Necrotic enteritis (**NE**) is an infection of the gastrointestinal tract and is estimated to cost the global poultry industry billions of dollars annually. A study was conducted to examine whether reducing the crude protein might offset the severity of NE in broilers experimentally challenged with *Eimeria* spp. on day 9 and *Clostridium perfringens* on days 14 and 15. Furthermore, increasing the dietary amino acid (**AA**) density of the diet was also examined owing to identified benefits of improving performance compromised from low protein (**LP**) diets or NE. A 2 × 2 × 3 factorial arrangement of treatments at 6 replicates per treatment was used with 972 Ross 308 cockerels fed wheat-sorghum-soy-based diets to 35 D. Factors were NE challenge: no or yes; protein: standard (**SP**) or LP; and AA density: 100% AA, 115% with only essential AA (**115% EAA**) increased, and 115% AA with both essential and nonessential AA (**115% AA**) increased. The performance was measured in grower (days 7–21), finisher (days 21–35), and overall (day 7–35) periods. In addition, on day 16, intestinal lesion score and cecal short-chain fatty acids were measured. Only in nonchallenged birds fed LP diets, 115% AA increased grower feed intake ($P < 0.01$) and body weight gain ($P < 0.05$) compared to 115% EAA treatments. Challenge increased jejunal lesions ($P < 0.001$) with no difference between dietary treatments. Finisher body weight gain was greater in nonchallenged birds fed the 115% AA diets than in challenged birds ($P < 0.05$). Feeding diets with higher nonessential AA encouraged faster recovery from NE challenge. When fed the SP diets, NE challenge increased cecal butyric acid ($P < 0.01$) and total short-chain fatty acids ($P < 0.05$). The nutrient matrix used in LP diets does not favor beneficial butyric acid–producing bacteria. Using LP diets to mitigate NE severity does not offset the predisposing effect of *E.* spp. when attacking the gastrointestinal tract, and NE recovery is favored when feeding SP diets or additional AA.

Key words: poultry nutrition, amino acid densities, low protein, necrotic enteritis, broiler chicken

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INTRODUCTION

High dietary protein has been associated with contributing to the proliferation of harmful bacteria such as *Clostridium perfringens* (*C. perfringens*) in the gut (Drew et al., 2004). NetB toxin–producing *C. perfringens* is a pathogenic bacteria found in the gastrointestinal tract (**GIT**) of chickens that can cause necrotic enteritis (**NE**), an infection of the GIT believed to cause the global poultry industry between USD $2 to 6 billion annually in losses (Dahiya et al., 2006; Wade and Keyburn, 2015). Acute NE is identified by lesions in the intestinal wall, particularly the jejunum (Cooper et al., 2013). In clinical cases, NE symptoms include depression, diarrhea, decreased appetite, and death (Ficken and Wages, 1997).

Currently, the use of in-feed antibiotic growth promoters (**AGP**) in animal feed is banned in the European Union with predictions for further restrictions extending into other regions. This has prompted the investigation of AGP replacements and revised feeding strategies to help maintain the performance standards currently achieved by feeding diets with AGP. Nutritional strategies such as reducing dietary protein and refining dietary amino acid (**AA**) profiles may be beneficial to prevent NE as *C. perfringens* cannot synthesize many AA and is dependent on the AA in its environment for function

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and reproduction. Shimizu et al., (2002) found many genes absent in *C. perfringens* that are responsible for the synthesis of arginine, aromatic AA, branched-chain AA, glutamic acid, histidine, lysine, methionine, serine, and threonine. Therefore, in higher crude protein (**CP**) diets, excess protein enters the hindgut where *C. perfringens* resides resulting in proliferation and increasing the risk of infection (Drew et al., 2004). In addition, when reducing dietary CP, the fiber content of the diet increases but increasing dietary fiber benefits the microbiome by promoting beneficial bacteria (Apajalahti et al., 2004). Supporting the proliferation of these beneficial bacteria increases competition for lumen space and mitigates the proliferation of harmful bacteria (Bao and Choct, 2010). However, when formulating low protein (**LP**) diets, less soy nonstarch polysaccharides (**NSPs**) are included creating a possible shortfall in using LP diets as a nutritional strategy to negate the occurrence of NE.

Feeding LP diets has additional environmental benefits by reducing N pollution and water consumption leading to improvements in litter quality and associated health and welfare outcomes (Alleman and Leclercq, 1997; Powers and Angel, 2008). Reducing CP in broiler diets has however resulted in reduced body weight gain (**BWG**) and increased feed conversion ratio (**FCR**) (Bregendahl et al., 2002). These effects have been mediated with crystalline essential AA supplements including glycine (**Gly**) equivalents ($\mathbf{Gly_{equiv}}$) (Dean et al., 2006). In addition, feeding birds ideal AA ratios in LP diets have demonstrated the ability to maintain BWG and FCR similar to that observed with standard protein (**SP**) diets (Belloir et al., 2017). The use of AA supplements has enabled the reduction of CP in research studies and demonstrated reductions in environmental pollution from broiler production.

To accrete protein, birds should be fed sufficient AA to satisfy maintenance and growth. The levels recommended by breeding companies have defined concentrations to meet this; however, protein accretion can be increased with alterations to these guidelines. Increasing AA densities have shown to increase BWG and decrease FCR compared to NRC recommendations (Kidd et al., 2004; Cloft et al., 2019). Furthermore, Keerqin et al. (2017) identified benefits of increasing AA density in the starter diet and negating the performance reducing effects of subclinical NE. In that study, increasing dietary standardized ileal digestible (**SID**) AA to meet the increased demand from inflammatory and immune response was found to facilitate recovery. However, when increasing the AA density of the diet, the CP also increased, and therefore, the benefits may have been attributed to other protein-related nutrients including nonessential AA rather than higher essential AA alone. Therefore, the effects of reducing CP and increasing AA density during subclinical NE challenge should be investigated to better prepare the industry for the extension of LP and AGP-free diets.

MATERIALS AND METHODS

Experimental Design and Diets

Ross 308 cockerels were sourced (n = 972) from Aviagen breeder hatchery in Goulburn, NSW, Australia. Chickens were raised in 3 temperature-controlled rooms at the Rob Cummings Poultry Innovation Centre at the University of New England, Armidale, Australia. All experimental procedures were approved by the Animal Ethics Committee (AEC18-059). On day 7, birds were weighed and assigned to 72 equal-sized floor pens (120 × 75 cm) based on approximate equal pen weight within 5% variation of the mean and checked for no significant differences. Birds were raised on clean wood shavings as the bedding material and each pen was assigned to one of 6 dietary treatments replicated 12 times as shown in Tables 1 and 2. Six replicates of each dietary treatment were selected for NE challenge at 14 birds per pen and the remaining 6 were assigned as nonchallenged at 13 birds per pen. Challenge groups were partitioned to minimize the spread of disease to nonchallenged birds. No room effect was observed. Until day 7, all birds were fed the same starter diet (3,000 kcal/kg, 24.6% CP) containing wheat, sorghum, and soybean meal. The treatments were arranged in a 2 × 2 × 3 factorial with factors as follows: NE challenge (no or yes), dietary CP level (SP or LP), and AA densities (**100% AA**, **115% EAA,** and **115% AA**). The AA densities were formulated using Evonik AMINOChick 2.0 software (Evonik Animal Nutrition, 2016), with essential AA multiplied by 115% to give 115% EAA and 115% AA treatments. To formulate 115% EAA treatments, AA requirements were met with crystalline AA supplements only and the CP was maintained to be identical to the 100% AA treatment for that respective CP level. To formulate 115% AA, both crystalline AA and CP were increased to achieve the higher AA requirements resulting in diets with greater nonessential AA levels than those in 100% and 115% EAA treatments. Diets were pelleted and isoenergetic at 3,080 and 3,100 kcal/kg for the grower (days 7–21) and finisher (days 21–35) treatments, respectively. The SP treatment with 100% AA represented an industry-standard diet using the crystalline AA; D,L-methionine, L-lysine HCl, L-threonine, and L-valine. The LP diet was formulated using all essential crystalline AA including L-isoleucine, L-arginine, L-phenylalanine, L-histidine, and L-tryptophan as well as glycine. The diets were isocaloric across all treatments. Glycine equivalents were determined following the equation glycine + (0.7143 × serine) (Dean et al., 2006) and formulated to 1.6% digestible $\mathrm{Gly_{equiv}}$ following previous findings from this group (Hilliar et al., 2019). Potassium carbonate was used to maintain a similar dietary electrolyte balance (≤237) between treatments following recommendations by Murakami et al. (2003).

The diets were based on wheat, sorghum, soybean meal, and canola oil. The nutrient profile of raw ingredients was determined using near-infrared reflectance

Table 1. Ingredients and nutrient content of grower (days 7–21) experimental diets.

Ingredients %	SP 100% AA	LP 100% AA	SP 115% EAA	LP 115% EAA	SP 115% AA	LP 115% AA
Wheat (10.4% CP)	49.67	63.94	52.68	63.31	42.19	55.73
Soybean meal (46.2% CP)	31.25	9.27	26.24	2.55	39.53	22.27
Sorghum (11.1% CP)	12.45	15.45	13.20	15.85	10.60	13.90
Canola oil	3.42	1.30	2.55	1.54	4.48	1.90
Alpha cellulose	0.00	2.05	0.00	5.00	0.00	0.00
Dicalcium phosphate	0.479	0.647	0.519	0.732	0.433	0.546
Limestone	0.984	1.021	0.992	1.020	0.964	1.000
Sodium chloride	0.177	0.157	0.172	0.152	0.185	0.169
Sodium bicarbonate	0.150	0.150	0.150	0.150	0.150	0.150
Potassium carbonate	0.000	0.410	0.000	0.672	0.000	0.000
Xylanase[1]	0.005	0.005	0.005	0.005	0.005	0.005
Phytase[2]	0.010	0.010	0.010	0.010	0.010	0.010
Titanium dioxide	0.500	0.500	0.500	0.500	0.500	0.500
Vitamin premix[3]	0.060	0.060	0.060	0.060	0.060	0.060
Mineral premix[4]	0.100	0.100	0.100	0.100	0.100	0.100
L-Lys SO_4	0.282	1.234	0.809	1.861	0.246	0.978
D,L-Met	0.315	0.468	0.471	0.657	0.388	0.497
L-Thr	0.092	0.370	0.267	0.582	0.106	0.316
L-Val	0.030	0.391	0.258	0.670	0.052	0.320
Gly	0.000	0.553	0.326	0.963	0.007	0.423
L-Ile	0.000	0.348	0.201	0.594	0.000	0.262
L-Arg	0.000	0.359	0.109	0.749	0.000	0.205
L-Leu	0.000	0.558	0.254	0.943	0.000	0.363
L-His	0.000	0.101	0.004	0.236	0.000	0.039
L-Phe	0.000	0.403	0.077	0.854	0.000	0.197
L-Trp	0.000	0.035	0.000	0.103	0.000	0.005
Choline chloride	0.025	0.105	0.044	0.141	0.000	0.057
Nutrients						
AMEn, kcal/kg	3,080	3,080	3,080	3,080	3,080	3,080
CP	21.6	17.1	21.6	17.1	24.5	20.9
SID[5] Met	0.573	0.636	0.708	0.787	0.675	0.718
SID TSAA	0.840	0.840	0.960	0.960	0.960	0.960
SID Lys	1.130	1.130	1.300	1.300	1.300	1.300
SID Thr	0.720	0.720	0.830	0.830	0.830	0.830
SID Val	0.890	0.890	1.030	1.030	1.030	1.030
SID Gly	0.727	0.979	0.982	1.274	0.836	1.026
SID Gly_{equiv}	1.619	1.513	1.791	1.674	1.850	1.773
SID Ile	0.780	0.780	0.900	0.900	0.900	0.900
SID Arg	1.239	1.180	1.350	1.350	1.457	1.350
SID Leu	1.410	1.210	1.390	1.390	1.598	1.390
SID His	0.473	0.370	0.430	0.430	0.544	0.430
SID Phe	0.916	0.928	0.904	1.233	1.050	0.954
SID Phe + Tyr	1.589	1.310	1.510	1.510	1.824	1.510
SID Trp	0.245	0.180	0.222	0.210	0.279	0.210
Calcium	0.750	0.750	0.750	0.750	0.750	0.750
Available phosphorus	0.380	0.380	0.380	0.380	0.380	0.380
Sodium	0.177	0.172	0.175	0.169	0.178	0.175
Potassium	0.963	0.790	0.869	0.790	1.104	0.798
Chloride	0.160	0.160	0.160	0.160	0.160	0.160
Choline	1,600	1,600	1,600	1,600	1,600	1,600
Linoleic acid	1.757	1.200	1.532	1.200	2.003	1.373
DEB[6] mEq/kg	279	237	255	237	314	238

Abbreviations: AA, amino acids; AMEn, apparent metabolizable energy corrected for nitrogen; Arg, arginine; CP, crude protein; DEB, dietary electrolyte balance; EAA, essential amino acids; Gly, glycine; Gly_{equiv}, glycine equivalence; His, histidine; Leu, leucine; Ile, isoleucine; LP, low protein; Lys, lysine; Met, methionine; Phe, phenylalanine; SP, standard protein; Thr, threonine; Trp, tryptophan; TSAA, total sulfur amino acids; Tyr, tyrosine; Val, valine.

[1]Econase XT 25 (AB Vista, 1000 BXU/kg).

[2]Quantum Blue, 5 G (AB Vista, 500 FTU/kg).

[3]Vitamin premix per kg diet: vitamin A, 12 MIU; vitamin D, 5 MIU; vitamin E, 75 mg; vitamin K, 3 mg; nicotinic acid, 55 mg; pantothenic acid, 13 mg; folic acid, 2 mg; riboflavin, 8 mg; cyanocobalamin, 0.016 mg; biotin, 0.25 mg; pyridoxine, 5 mg; thiamine, 3 mg; antioxidant, 50 mg.

[4]Mineral premix per kg diet: Cu, 16 mg as copper sulfate; Mn, 60 mg as manganese sulfate; Mn, 60 mg as manganous oxide; I, 0.125 mg as potassium iodide; Se, 0.3 mg; Fe, 40 mg, as iron sulfate; Zn, 50 mg as zinc oxide; Zn, 50 mg as zinc sulfate.

[5]Digestible coefficients for raw ingredients determined using AMINODat 5.0 (Evonik Animal Nutrition).

[6]DEB mEq/kg calculated as $10{,}000 \times (Na^+ + K^+ - Cl^-)$.

spectroscopy (Foss NIR 6500, Denmark) standardized to Evonik AMINONIR Advanced calibration. Exogenous enzymes xylanase (Econase XT 25, AB Vista) and phytase (Quantum Blue, 5 G, AB Vista) were added to the diets at 1000 BXU/kg and 500 FTU/kg, respectively. Manufacturer-recommended AA matrix values for phytase were removed from diet formulations owing to variability between diets of phytate mainly from differences in soybean meal inclusion levels. To determine apparent ileal N digestibility, titanium dioxide was used as an inert marker and formulated into the diet at 0.5%.

Table 2. Ingredients and nutrient content of finisher (day 21–35) experimental diets.

Ingredients %	SP 100% AA	LP 100% AA	SP 115% EAA	LP 115% EAA	SP 115% AA	LP 115% AA
Wheat (10.4% CP)	54.24	68.66	56.60	68.54	47.41	61.16
Soybean meal (46.2% CP)	26.38	6.92	22.44	0.87	33.96	15.97
Sorghum (11.1% CP)	13.60	17.20	14.20	17.10	11.90	15.30
Canola oil	2.97	0.32	2.24	0.54	3.94	1.42
Alpha cellulose	0.00	0.00	0.00	2.65	0.00	0.00
Dicalcium phosphate	0.285	0.418	0.316	0.501	0.242	0.369
Limestone	0.933	0.971	0.939	0.970	0.915	0.951
Sodium chloride	0.177	0.159	0.174	0.155	0.185	0.168
Sodium bicarbonate	0.150	0.150	0.150	0.150	0.150	0.150
Potassium carbonate	0.000	0.606	0.000	0.841	0.000	0.330
Xylanase[1]	0.005	0.005	0.005	0.005	0.005	0.005
Phytase[2]	0.010	0.010	0.010	0.010	0.010	0.010
Titanium dioxide	0.500	0.500	0.500	0.500	0.500	0.500
Vitamin premix[3]	0.050	0.050	0.050	0.050	0.050	0.050
Mineral premix[4]	0.100	0.100	0.100	0.100	0.100	0.100
L-Lys SO_4	0.247	1.077	0.692	1.636	0.204	0.969
D,L-Met	0.261	0.387	0.400	0.559	0.328	0.443
L-Thr	0.078	0.316	0.229	0.508	0.090	0.309
L-Val	0.008	0.315	0.198	0.561	0.019	0.301
Gly	0.000	0.370	0.167	0.728	0.000	0.327
L-Ile	0.000	0.288	0.174	0.522	0.000	0.274
L-Arg	0.000	0.474	0.212	0.827	0.000	0.391
L-Leu	0.000	0.241	0.172	0.593	0.000	0.190
L-His	0.000	0.073	0.000	0.190	0.000	0.044
L-Phe	0.000	0.291	0.000	0.687	0.000	0.197
L-Trp	0.000	0.020	0.000	0.094	0.000	0.022
Choline chloride	0.020	0.086	0.035	0.118	0.000	0.058
Nutrients						
AMEn, kcal/kg	3,100	3,100	3,100	3,100	3,100	3,100
CP	19.8	15.8	19.8	15.8	22.5	18.6
SID[5] Met	0.504	0.554	0.625	0.692	0.597	0.643
SID TSAA	0.760	0.760	0.870	0.870	0.870	0.870
SID Lys	1.000	1.000	1.150	1.150	1.150	1.150
SID Thr	0.650	0.650	0.750	0.750	0.750	0.750
SID Val	0.800	0.800	0.921	0.920	0.920	0.920
SID Gly	0.668	0.781	0.780	1.037	0.761	0.853
SID Gly_{equiv}	1.489	1.297	1.536	1.433	1.694	1.507
SID Ile	0.710	0.700	0.820	0.820	0.820	0.820
SID Arg	1.111	1.050	1.211	1.210	1.311	1.210
SID Leu	1.301	1.070	1.371	1.230	1.473	1.230
SID His	0.432	0.330	0.396	0.380	0.497	0.380
SID Phe	0.838	0.795	0.768	1.060	0.961	0.852
SID Phe + Tyr	1.452	1.160	1.330	1.330	1.668	1.330
SID Trp	0.225	0.160	0.207	0.200	0.256	0.200
Calcium	0.680	0.680	0.680	0.680	0.680	0.680
Available phosphorus	0.340	0.340	0.340	0.340	0.340	0.340
Sodium	0.177	0.173	0.176	0.170	0.178	0.174
Potassium	0.881	0.878	0.807	0.878	1.010	0.878
Chloride	0.160	0.160	0.160	0.160	0.160	0.160
Choline	1,500	1,500	1,500	1,500	1,513	1,500
Linoleic acid	1.657	1.000	1.470	1.000	1.884	1.263
DEB[6] mEq/kg	258	259	239	259	290	258

Abbreviations: AA, amino acids; AMEn, apparent metabolizable energy corrected for nitrogen; Arg, arginine; CP, crude protein; DEB, dietary electrolyte balance; EAA, essential amino acids; Gly, glycine; Gly_{equiv}, glycine equivalence; His, histidine; Leu, leucine; Ile, isoleucine; LP, low protein; Lys, lysine; Met, methionine; Phe, phenylalanine; SP, standard protein; Thr, threonine; Trp, tryptophan; TSAA, total sulfur amino acids; Tyr, tyrosine; Val, valine.

[1]Econase XT 25 (AB Vista, 1000 BXU/kg).

[2]Quantum Blue, 5 G (AB Vista, 500 FTU/kg).

[3]Vitamin premix per kg diet: vitamin A, 12 MIU; vitamin D, 5 MIU; vitamin E, 75 mg; vitamin K, 3 mg; nicotinic acid, 55 mg; pantothenic acid, 13 mg; folic acid, 2 mg; riboflavin, 8 mg; cyanocobalamin, 0.016 mg; biotin, 0.25 mg; pyridoxine, 5 mg; thiamine, 3 mg; antioxidant, 50 mg.

[4]Mineral premix per kg diet: Cu, 16 mg as copper sulfate; Mn, 60 mg as manganese sulfate; Mn, 60 mg as manganous oxide; I, 0.125 mg as potassium iodide; Se, 0.3 mg; Fe, 40 mg, as iron sulfate; Zn, 50 mg as zinc oxide; Zn, 50 mg as zinc sulfate.

[5]Digestible coefficients for raw ingredients determined using AMINODat 5.0 (Evonik Animal Nutrition).

[6]DEB mEq/kg calculated as $10{,}000 \times (Na^+ + K^+ - Cl^-)$.

Necrotic Enteritis Challenge

Challenge with subclinical NE followed the modified method described by Rodgers et al. (2015). On day 9, all challenged chicks were inoculated *per os* 1 mL of suspension containing 5,000 sporulated oocysts of *Eimeria acervulina*, and *Eimeria maxima*, and 2,500 sporulated oocysts of *Eimeria brunetti* (Eimeria Pty Ltd., Werribee Victoria, Australia). All control birds were inoculated with 1 mL sterile phosphate buffer solution to mimic inoculation stressors. On days 14 and 15, challenged birds were inoculated *per os* with 1 mL of 10^8 CFU/mL *C perfringens* (EHE-NE18; CSIRO Livestock Industries,

Geelong, Australia) suspension in a thioglycolate broth that had been incubated overnight at 39°C. All nonchallenged birds were inoculated *per os* with 1 mL of sterile thioglycolate broth. On day 16, 2 birds per pen were electrically stunned and sacrificed for a necropsy. Duodenum, jejunum, and ileum were examined for intestinal lesions by experienced personnel blind to the trial design following techniques described by Keyburn et al. (2006) and scored from 0 to 6 on lesion severity.

Performance Measurements and Sampling

Weekly weights of chickens and feeders were recorded from days 7 to 35 to determine total feed intake (**FI**) and pen body weight. To determine FCR, total FI was divided by the BWG with correction for mortalities. Average FI was then calculated by multiplying BWG by FCR. Three and 2 birds were randomly selected on day 16 and 35, respectively, and sacrificed by electrical stunning and dissection for sample collection. Cecal contents were collected on day 16, and ileal digesta and blood serum were collected on both day 16 and 35. Cecal contents were collected for short-chain fatty acid analysis and ileal digesta were collected for N digestibility. The small intestine between the Meckel's diverticulum and 1 cm before the ileocecal junction was defined as the ileum. Ileal contents were collected by cutting along the ileum to avoid contaminating samples with intestinal secretions.

Diet and Digesta Analysis

The finished feed was subsampled, and AA and tryptophan analysis were completed by AMINOLAB (Singapore, Evonik SEA). Amino acids were analyzed using standard procedures (AOAC, 1994) with an AA analyzer (Biochrom 30+, Cambridge, UK). Tryptophan was measured in feed samples by high-performance liquid chromatography. Digesta was stored at −20°C until freeze-drying using the Christ Alpha 1-3 LDplus freeze dryer (Osterode am Harz, Germany). To determine feed and digesta CP, samples were grounded and analyzed for N by combustion (LECO Corp., St. Joeseph, MI) using standard procedures, and N content was then multiplied by 6.25. Titanium dioxide in diets and digesta was measured following Short et al. (1996) in duplicate by colorimetric method.

Ileal pH

To measure ileal pH on d 16, a digital pH meter (Ecoscan, Eutech Instrument, Singapore) fitted with a spear-tip pH electrode (Sensorex S175 C) was used by inserting into the digesta of the proximal-, mid-, and distal-ileum (Morgan et al., 2014). Two readings were taken from each section and averaged, and the probe was washed with deionized water between sections and samples.

Serum Creatine Kinase

Blood was collected from 2 birds per pen on d 16 and pooled in Vacutainers (Beckton Dickinson, North Ryde, NSW, Australia) that contained spray-coated silica and a polymer gel for serum separation and stored at 4°C until centrifuged. The serum samples were centrifuged at 1,500 × g at 4°C for 5 min to separate the serum and stored at −20°C until analysis. Serum was analyzed for serum creatine kinase (**SCK**) activity using an integrated chemistry analyzer (Siemens Dimension Xpand Plus, Newark, NJ, USA).

Succinic Acid, Lactic Acid, and Short-Chain Fatty Acid Analysis

Cecal contents were pooled per pen on day 16 for succinic acid, lactic acid, and short-chain fatty acids (**SCFA**) analysis. Samples were stored at −20°C and thawed for analysis. Samples were analyzed in duplicate following methods described by Wu et al. (2010).

Statistical Analysis

All normal data were subject to a general linear model using a 2 × 2 × 3 factorial arrangement with means separated at $P < 0.05$ using the Tukey post hoc test (Minitab v. 17.1.0). Non-normal SCFA data were log-transformed before undergoing the same statistical analysis. Non-normal livability and lesion scoring data were subjected to Kruskal–Wallis nonparametric tests and means were separated when $P < 0.05$.

RESULTS

Diets and Performance

Grower and finisher treatment diet analysis for CP and total AA (Supplemental Tables 1 and 2) is consistent with calculated values.

Performance data are shown in Table 3. A three-way challenge × CP × AA interaction was observed for grower (days 7–21) FI ($P < 0.05$). In only challenged birds fed the LP diet, feeding 115% AA increased FI (by 12.4%) compared to nonchallenged birds fed the 115% EAA treatment. A three-way challenge × CP × AA interaction was observed for grower (days 7–21) BWG ($P < 0.01$) as well. Only in nonchallenged birds fed the LP diet, 115% EAA reduced BWG compared to birds fed 100% AA and 115% AA by 11.3 and 16.9%, respectively. Grower FCR was affected by challenge ($P < 0.001$), protein ($P < 0.01$), and AA ($P < 0.01$) as main effects without interactions observed. Challenged birds had 25-points higher FCR than nonchallenged, feeding LP diets increased FCR by 6 points compared to SP diets, and feeding 115% AA reduced FCR by 8 points compared to 100% AA treatments.

Table 3. Performance results for grower (day 7 to 21), finisher (day 21 to 35), and overall periods (day 7 to 35).

			Start	Grower (day 7 to 21)			Finisher (day 21 to 35)			Overall (day 7 to 35)		
Challenge	Protein	AA	Weight (g)	FI (g)	BWG (g)	FCR (g/g)	FI (g)	BWG (g)	FCR (g/g)	FI (g)	BWG (g)	FCR (g/g)
No	SP	100% AA	171	1167^{a}	941^{a}	1.241	2,505	1,573	1.593	3,446	2515^{b,c}	1.443
		115% EAA	175	1155^{a,b}	967^{a}	1.195	2,566	1,603	1.604	3,533	2570^{b,c}	1.410
		115% AA	174	1132^{a,b}	942^{a}	1.204	2,633	1,865	1.412	3,575	2807^{a}	1.330
	LP	100% AA	170	1157^{a,b}	894^{a}	1.297	2,470	1,404	1.761	3,364	2298^{d,e}	1.560
		115% EAA	174	1048^{b,c}	793^{b}	1.321	2,169	1,354	1.601	2,963	2148^{e,f,g}	1.480
		115% AA	175	1178^{a}	954^{a}	1.237	2,552	1,709	1.493	3,507	2663^{a,b}	1.382
Yes	SP	100% AA	174	963^{c}	632^{c,d}	1.534	2,282	1,443	1.584	2,914	2075^{f,g}	1.566
		115% EAA	174	947^{c}	647^{c,d}	1.465	2,296	1,570	1.463	2,943	2217^{d,e,f}	1.463
		115% AA	177	1011^{c}	702^{c}	1.437	2,491	1,719	1.450	3,193	2422^{c,d}	1.445
	LP	100% AA	175	960^{c}	617^{c,d}	1.557	2,394	1,486	1.611	3,012	2104^{e,f,g}	1.592
		115% EAA	176	955^{c}	611^{d}	1.566	2,068	1,368	1.512	2,679	1979^{g}	1.529
		115% AA	171	954^{c}	665^{c,d}	1.437	2,284	1,514	1.510	2,949	2178^{e,f,g}	1.483
SEM			0.58	13.00	17.80	0.019	27.50	21.00	0.014	40.40	32.50	0.011
Main effects												
Challenge		No	173	1,140	915	1.249^{b}	2483^{a}	1,585	1.577	3398^{a}	2,500	1.434^{b}
		Yes	175	965	646	1.499^{a}	2303^{b}	1,517	1.522	2948^{b}	2,162	1.513^{a}
Protein		SP	174	1,062	805	1.346^{b}	2,462	1629^{a}	1.518^{b}	3,267	2,434	1.443^{b}
		LP	174	1,042	756	1.403^{a}	2,323	1473^{b}	1.581^{a}	3,079	2,228	1.504^{a}
AA density		100% AA	173	1,062	771	1.407^{a}	2,413	1477^{b}	1.637	3,184	2,248	1.540^{a}
		115% EAA	175	1,026	754	1.387^{a,b}	2,275	1474^{b}	1.545	3,029	2,229	1.471^{b}
		115% AA	174	1,069	816	1.329^{b}	2,490	1702^{a}	1.466	3,306	2,518	1.410^{c}
P-value												
Challenge			0.246	<0.001	<0.001	<0.001	<0.001	0.005	0.003	<0.001	<0.001	<0.001
Protein			0.725	0.160	<0.001	0.009	0.001	<0.001	0.001	<0.001	<0.001	<0.001
AA			0.261	0.040	<0.001	0.009	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Challenge × protein			0.767	0.825	0.063	0.473	0.440	0.134	0.310	0.248	0.037	0.142
Challenge × AA			0.369	0.368	0.263	0.482	0.851	0.010	0.005	0.948	0.024	0.167
Protein × AA			0.403	0.354	0.002	0.147	0.004	0.018	0.242	0.001	0.002	0.625
Challenge × protein × AA			0.176	0.011	0.002	0.996	0.261	0.073	0.097	0.075	0.018	0.288

[a-e]Differing superscripts within column group indicate significant differences between means by general linear model with post hoc Tukey test ($P < 0.05$).

Abbreviations: AA, amino acids; BWG, body weight gain; EAA, essential amino acids; FCR, feed conversion ratio; FI, feed intake; LP, low protein; SP, standard protein.

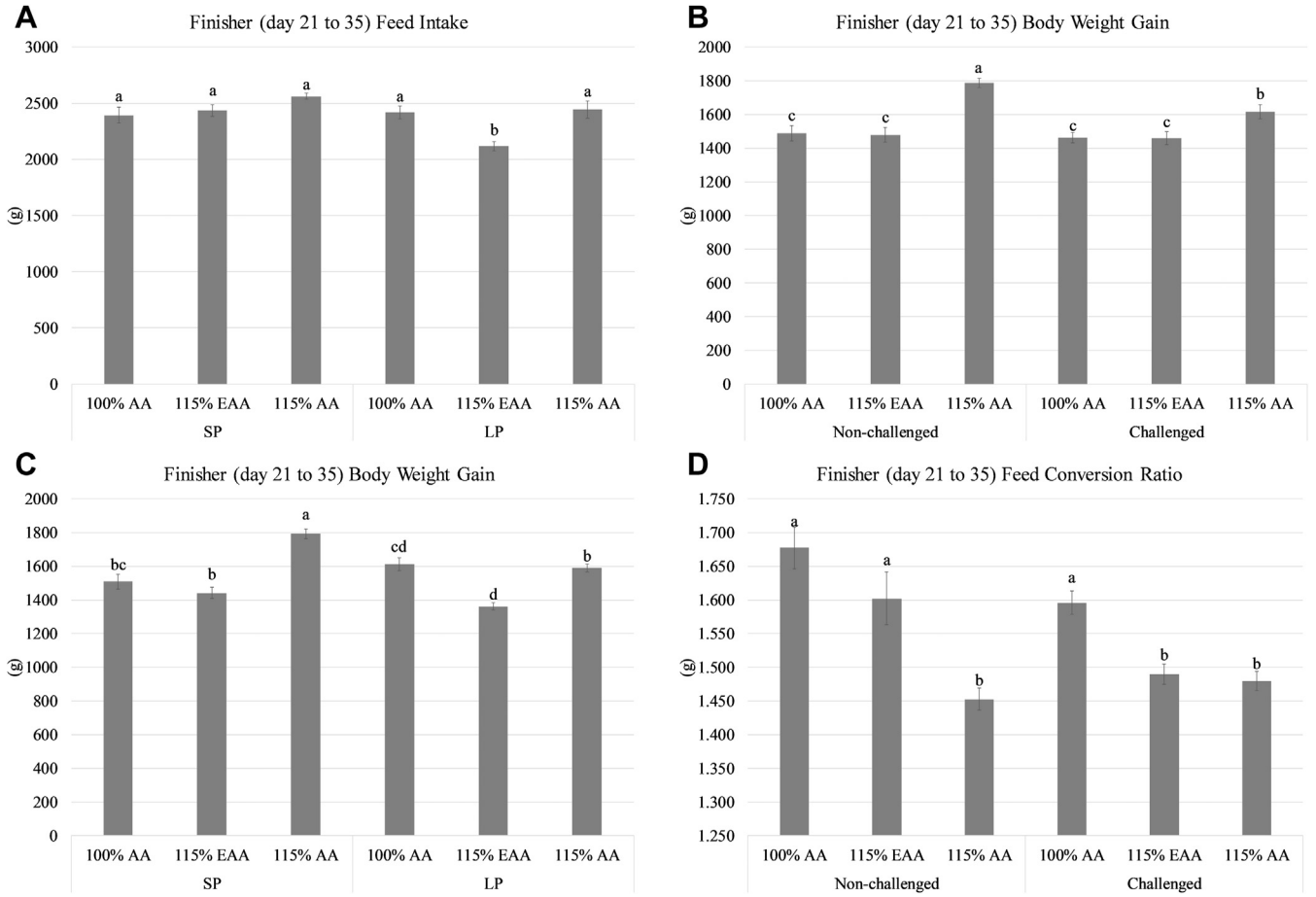

Figure 1. Finisher performance data two-way interactions: (A) Crude protein × amino acid density two-way interactions for finisher (day 21–35) feed intake ($P < 0.01$). (B) Challenge × amino acid density two-way interactions for finisher (day 21–35) body weight gain ($P < 0.05$). (C) Protein × amino acid density interactions for finisher (day 21–35) body weight gain ($P < 0.05$). (D) Challenge × amino acid density interactions for finisher (day 21–35) feed conversion ratio ($P < 0.01$). [a,b,c,d]Differing superscripts indicate significant differences between means by general linear model with post hoc Tukey test ($P < 0.05$). Abbreviations: SP, standard protein; LP, low protein; AA, amino acids; EAA, essential amino acids.

A CP × AA interaction ($P < 0.01$) occurred in finisher (days 21–35) FI, as shown in Figure 1A. Birds fed the LP diet with 115% EAA had an FI of 2,119 g, 13.0% lower than birds fed the SP diet with 115% EAA (2,436 g), and 12.4 and 13.3% less than birds fed the LP diets with 100% AA (2,418 g) and 115% AA, respectively (2,443 g). An NE challenge main effect was also observed ($P < 0.001$) in finisher (days 21–35) FI as challenged birds consumed 7.2% less feed than nonchallenged birds. A two-way interaction of challenge × AA ($P < 0.05$) occurred for finisher BWG as shown in Figure 1B. In nonchallenged birds, feeding 115% AA (1,787 g) increased BWG by 20.0 and 20.8% compared to 100% AA (1,489 g) and 115% EAA (1,479 g) treatments, respectively. In addition, within challenged birds, feeding 115% AA (1,616 g) increased weight gain by 9.5 and 10.7% compared to 100% AA (1,463 g) and 115% EAA (1,460 g) treatments, respectively. A CP × AA interaction also occurred for finisher (days 21–35) BWG ($P < 0.05$) as shown in Figure 1C. Feeding 115% AA in the SP diet (1,792 g) increased finisher (days 21–35) weight gain by 18.8 and 24.4% compared to SP diets with 100% AA (1,508 g) and 115% EAA (1,441 g) respectively. In addition, feeding the SP diets with 115% EAA or 115% AA increased finisher (days 21–35) BWG by 5.8 and 12.8% compared to the LP diets with 115% EAA (1,362 g) or 115% AA (1,588 g), respectively. As shown in Figure 1D, by a two-way challenge × AA interaction ($P < 0.01$), feeding 115% EAA in challenged birds (1.490 g/g) decreased finisher (days 21–35) FCR by 11 points compared to nonchallenged bird fed 115% EAA (1.602 g/g). In addition, in nonchallenged birds, a 115% AA diet (1.453 g/g) decreased finisher (days 21–35) FCR by 14 and 22 points compared to the nonchallenged birds fed 100% AA (1.677 g/g) and 115% EAA, respectively. In challenged birds, 115% EAA and 115% AA treatments reduced FCR by 11 and 12 points compared to birds fed 100% AA. Protein also had an independent effect on finisher (days 21–35) FCR as LP diets increased FCR by 6 points compared to birds fed SP treatments ($P < 0.01$).

A CP × AA interaction ($P < 0.01$) occurred in overall (day 7–35) FI as shown in Figure 2. Birds fed the LP diet with 115% EAA (2,821 g) consumed 12.0% less overall feed (days 7–35) than birds fed the SP diet with 115% EAA (3,204 g). In addition, those in the LP diet with 115% EAA reduced overall (days 7–35) FI by 12.6 and 13.6% compared to those fed LP diets with 100% AA

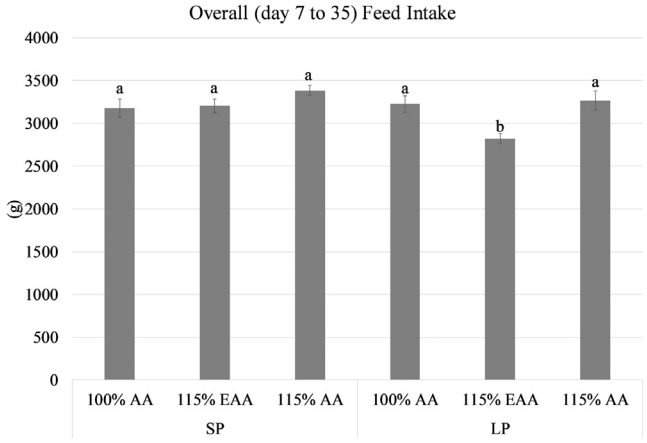

Figure 2. Protein × amino acid density two-way interactions for overall (day 7–35) feed conversion ratio ($P < 0.01$). [a,b,c]Differing superscripts indicate significant differences between means by general linear model with post hoc Tukey test ($P < 0.05$). Abbreviations: AA, amino acids; EAA, essential amino acids.

(3,228 g) and 115% AA (3,265 g), respectively. The main effect of challenge ($P < 0.001$) also resulted in a 13.2% decrease in overall FI (days 7–35) compared to nonchallenged birds. A three-way challenge × CP × AA interaction occurred for overall (days 7–35) BWG ($P < 0.05$). Only in nonchallenged birds fed 100% AA, did feeding the SP diet increase overall (days 7–35) BWG (by 9.4%) compared to birds fed the LP diet. Overall (days 7–35) FCR had significant differences in the main effects of challenge ($P < 0.001$), protein ($P < 0.001$), and AA ($P < 0.001$). Challenge increased overall (days 7–35) FCR by 8 points compared to nonchallenged birds. Feeding the LP diets increased overall (days 7–35) FCR by 6 points compared to birds fed the SP diets. Finally, feeding 115% EAA reduced overall (days 7–35) FCR by 7 points compared to birds fed 100% AA treatments and a further reduction of 6 points in overall (days 7–35) FCR when feeding 115% AA treatments compared to 115% EAA treatments. Livability was not affected during any phase of the experiment (Table 4).

Gastrointestinal Tract and Health

As shown in Figure 3, challenge treatments had a greater prevalence of jejunal lesions ($P < 0.001$). A challenge × CP interaction occurred in proximal ileal pH ($P < 0.05$) as shown in Figure 4A. Proximal ileal pH was lower in challenged birds fed the SP diets

Table 4. Day (D) 7 to 35 livability, ileal pH, serum creatine kinase, and apparent ileal nitrogen digestibility.

			Livability (%)	Ileal pH			SCK (U)	N dig (%)	
Challenge	Protein	AA	days 7–35	Prox	Mid	Dist	day 16	Grower	Finisher
No	SP	100% AA	94.9	6.34	6.49	6.46	3437^{a,b,c}	81.0	79.5^{a,b,c}
		115% EAA	91.0	6.31	6.21	6.02	3008^{a,b,c}	83.1	80.4^{a,b,c}
		115% AA	93.6	6.46	6.24	6.22	2927^{a,b,c}	85.0	81.7^{a,b}
	LP	100% AA	98.7	6.26	6.11	6.60	2473^{a,b,c}	81.5	82.4^{a}
		115% EAA	92.3	6.04	6.31	6.21	4210^{a,b}	82.9	80.5^{a,b,c}
		115% AA	87.2	5.94	5.91	5.84	4490^{a}	85.4	83.2^{a}
Yes	SP	100% AA	92.9	5.49	5.54	5.53	1570^{c}	69.4	72.3^{b,c,d}
		115% EAA	97.1	5.62	5.58	5.64	2723^{a,b,c}	66.8	64.1^{d,e}
		115% AA	92.9	5.65	5.88	5.54	2532^{a,b,c}	72.5	71.6^{c,d}
	LP	100% AA	91.4	5.88	5.57	5.37	2123^{c}	65.5	54.5^{f}
		115% EAA	94.1	6.17	5.66	5.67	1777^{c}	70.1	61.8^{e,f}
		115% AA	91.7	5.56	5.34	5.38	2347^{b,c}	70.1	66.2^{d,e}
SEM			0.85	0.08	0.08	0.08	155	0.99	1.22
Main effects									
Challenge		No	93.0	6.22	6.21^{a}	6.22^{a}	3,424	83.1^{a}	81.3
		Yes	93.3	5.73	5.60^{b}	5.52^{b}	2,179	69.1^{b}	65.1
Protein		SP	93.7	5.98	5.99	5.90	2,699	76.3	74.9
		LP	92.6	5.97	5.82	5.84	2,903	75.9	71.4
AA density		100% AA	94.5	5.99	5.93	5.99	2,401	74.4^{b}	72.2
		115% EAA	93.6	6.03	5.94	5.88	2,929	75.7^{a,b}	71.7
		115% AA	91.3	5.90	5.84	5.74	3,074	78.3^{a}	75.7
P value									
Challenge			0.393	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Protein			0.990	0.971	0.220	0.674	0.418	0.722	0.003
AA			0.505	0.730	0.821	0.318	0.075	0.011	0.013
Challenge × protein			0.596	0.049	0.829	0.757	0.118	0.558	<0.001
Challenge × AA			0.168	0.566	0.703	0.129	0.917	0.952	0.271
Protein × AA			0.842	0.328	0.311	0.492	0.342	0.402	0.055
Challenge × protein × AA			0.488	0.831	0.655	0.704	0.007	0.255	0.005

[a–e]Differing superscripts within column group indicate significant differences between means by general linear model with post hoc Tukey test ($P < 0.05$).

Abbreviations: AA, amino acids; Dist, distal; EAA, essential amino acids; LP, low protein; N dig = apparent ileal nitrogen digestibility; Prox, proximal; SCK, serum creatine kinase; SP, standard protein.

(5.59) compared to nonchallenged birds fed the SP diets (6.37). Ileal pH was reduced by the challenge ($P < 0.001$) in mid and distal sections of the ileum from 6.21 and 6.22 to 5.60 and 5.52, respectively, compared to nonchallenged birds.

Main effects of challenge ($P < 0.001$) and AA ($P < 0.05$) were observed in grower apparent ileal N digestibility. Challenging the birds reduced apparent ileal N digestibility by 14 percentage points during the grower phase and feeding 115% AA increased apparent ileal N digestibility 3.9 percentage points compared to 100% AA treatments. A three-way challenge × CP × AA interaction ($P < 0.01$) occurred for the finisher apparent ileal N digestibility. In only challenged birds fed the LP diet, feeding 115% AA increased finisher apparent ileal N digestibility by 11.7 percentage points compared to those fed 100% AA. A three-way challenge × protein × AA interaction occurred in SCK on d 16 ($P < 0.01$). Only in birds fed the LP diets with 115% EAA and 115% AA, NE challenge decreased SCK activity by 57.8 and 47.7%, respectively, compared to nonchallenged birds.

Two-way challenge × protein interactions were observed in cecal butyric acid ($P < 0.01$) and cecal total SCFA ($P < 0.05$) as shown in Figures 4B and 4C. In challenged birds, feeding SP diets (30.95 μmol/g) increased cecal butyric acid compared to those fed the LP diets (19.26 μmol/g), while in birds fed the SP diets, challenged birds (30.95 μmol/g) increased cecal butyric acid compared to nonchallenged birds (13.21 μmol/g). In addition, feeding challenged birds the SP diets (48.01 μmol/g) increased cecal total SCFA compared to challenged birds fed the LP diets (33.69 μmol/g) and nonchallenged birds fed the SP diets (34.73 μmol/g). Other volatile fatty acids such as formic acid ($P < 0.001$), acetic acid ($P < 0.001$), and succinic acid ($P < 0.001$) decreased as a result of NE challenge by 0.91, 16.26, and 9.08 μmol/g, respectively, compared to nonchallenged birds as shown in Table 5. In addition, isobutyric acid ($P < 0.01$), valeric acid ($P < 0.001$), and lactic acid ($P < 0.001$) increased by 0.29, 0.56, and 18.92 μmol/g in response to NE challenge, respectively, compared to nonchallenged birds as a main effect.

DISCUSSION

Necrotic enteritis challenge reduced weight gain, feed consumption, and feed efficiency compared to nonchallenged birds, similar to results observed by Xue et al., (2018) and Rodrigues et al., (2018) using the same challenge model. Appetite suppression is a symptom of GIT infections such as coccidiosis and NE which reduces weight gain and feed efficiency (Cooper et al., 2013). In the current experiment, the performance was reduced particularly during the grower period by challenge, reduced protein, and increased EAA density. Increasing CP and AA was hypothesized to induce severity in NE clinical symptoms by promoting the proliferation of *C. perfringens*. In nonchallenged birds, reducing CP typically results in reduced performance (Corzo et al., 2005; Dean et al., 2006; Ospina-Rojas et al., 2014); however, the diets were formulated to meet or exceed the essential AA requirements including Gly_{equiv}. In the present study, reducing CP reduced growth and development and exacerbated the effects of NE regardless of 15% additional essential AA. Therefore, the effects observed were not due to deficiencies in essential AA including Gly but most likely due to alterations in other nutrients associated with dietary CP such as nonessential AA or NSPs.

Dietary Gly inclusion has been associated with increasing cecal *C. perfringens* bacterial counts (Dahiya et al., 2005). To achieve a minimum Gly_{equiv} recommendation (Hilliar et al., 2019), Gly was supplemented. As a result, Gly was higher in LP diets compared to SP treatments and can be attributed to the similarity in FI and BWG between SP 100% AA and LP 100% AA treatments. Therefore, although supplementing Gly in LP diets is known to maintain performance, Gly may also contribute to the proliferation of *C. perfringens*. The reduced performance that

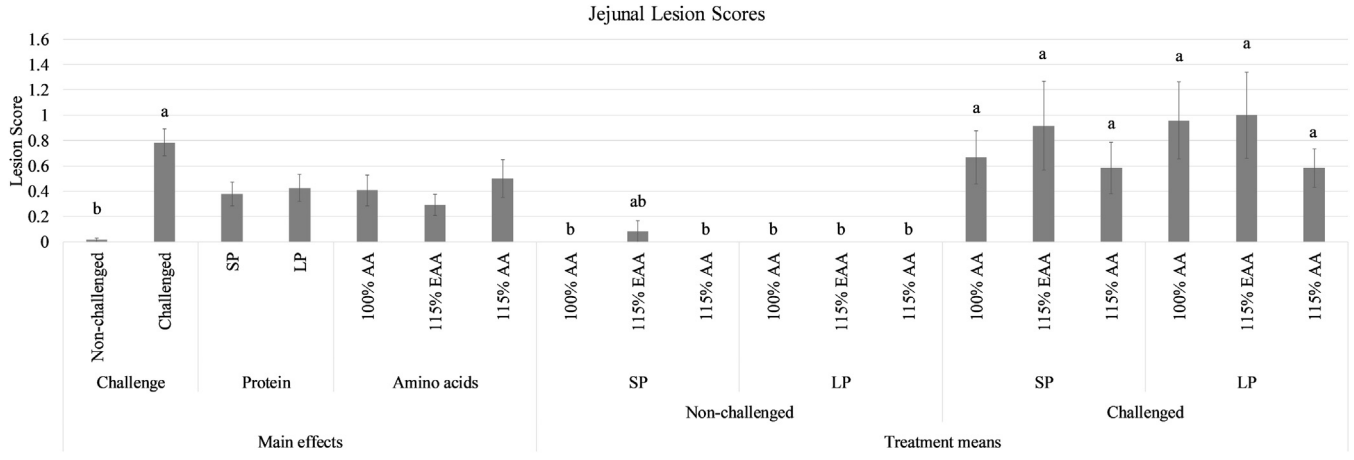

Figure 3. Challenge, protein, and amino acid main effects and treatment means for jejunal lesion scores. [a,b]Differing superscripts indicate significant differences between means by Kruskal–Wallis nonparametric test ($P < 0.05$). Abbreviations: SP, standard protein; LP, low protein; AA, amino acids; EAA, essential amino acids.

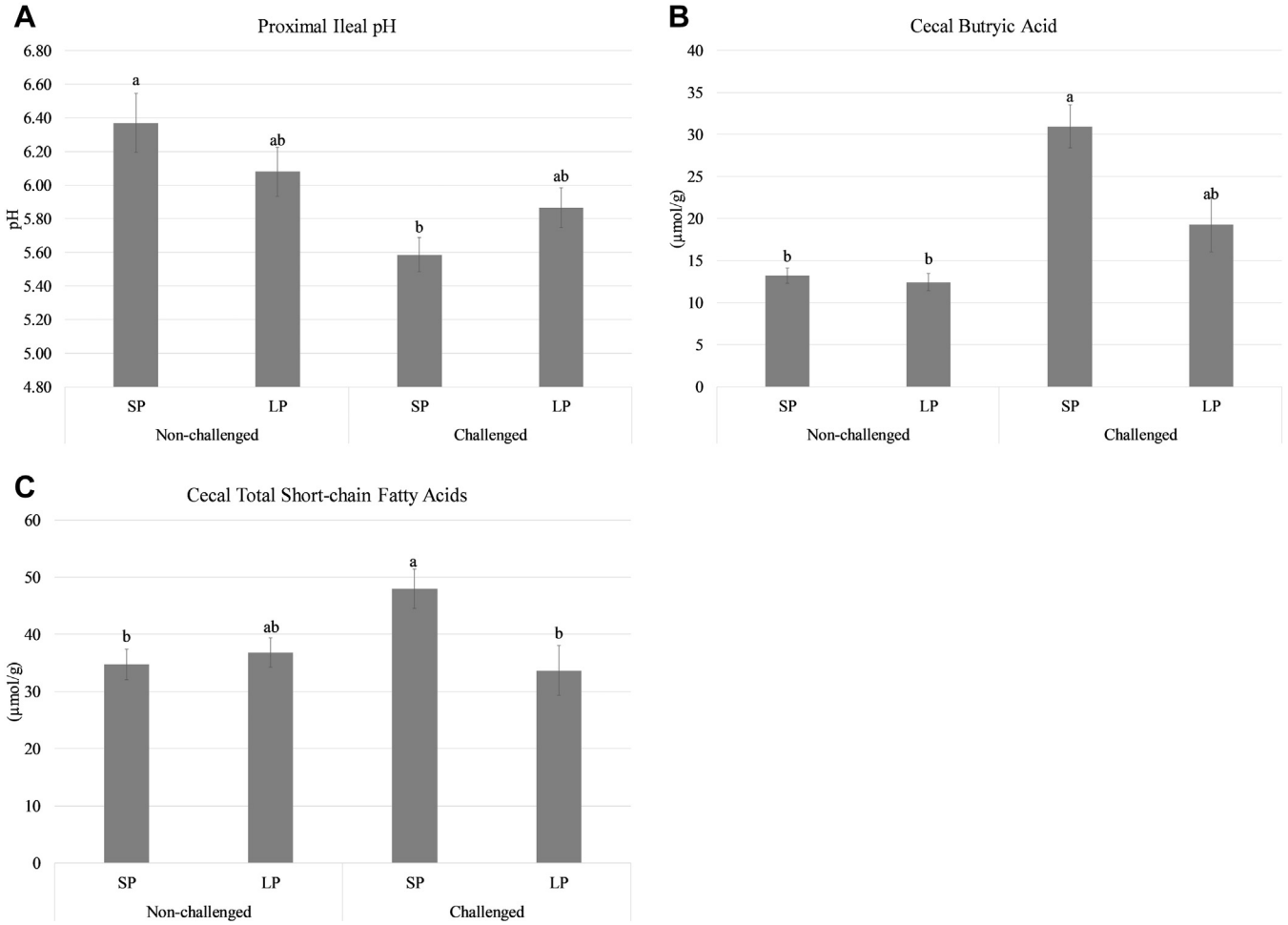

Figure 4. Challenge × crude protein two-way interactions for (A) proximal ileal pH ($P < 0.05$), (B) cecal butyric acid ($P < 0.01$), and (C) cecal total short-chain fatty acids ($P < 0.05$). [a,b]Differing superscripts indicate significant differences between means by general linear model with post hoc Tukey test ($P < 0.05$). Abbreviations: SP, standard protein; LP, low protein.

occurred in challenged birds fed the LP diets in the present study may be explained by the increased dietary Gly. However, the Gly levels in diets with 115% EAA and 115% AA were higher again than 100% AA diets, and no difference in performance was observed between challenged birds fed LP diets. This does not rule out the relationship identified by Dahiya et al. (2005) as the benefits of increasing the AA density on performance may outweigh the impacts of free Gly on the proliferation of *C. perfringens*. Increased AA density reduced performance differences between challenged and nonchallenged birds in their respective treatments during the finisher phase, enabling them to overcome the decreased performance of challenged birds during the grower period. The benefits of increasing AA density on growth and efficiency have been reported by Vieira and Angel (2012) and are supported by the findings of this study.

Increasing AA density in the diet as 115% AA increased finisher BWG in challenged birds and feeding either 115% EAA or 115% AA reduced finisher FCR compared to challenged birds fed 100% AA. Rochell et al. (2016) observed that reducing individual AA such as total sulfur AA (**TSAA**), lysine, threonine, valine, isoleucine, and arginine by 30% reduced performance in both nonchallenged and *E. acervulina* challenged birds. The effect observed by Rochell et al. (2016) in the treatments with lowering TSAA was greatest; however, AA deficiency resulted in lower BWG again with *E. acervulina* challenge. In the present study, reducing nonessential AA in the diet had a similar effect as performance deteriorated in nonchallenged birds and challenged birds fed higher CP diets. This highlights the roles of nonessential AA on GIT development. Increasing the AA density may benefit gut health and development. Barekatain et al. (2019) found increasing essential AA by 10% on Ross 308 breed standards (Aviagen, 2014) improved gut integrity. Furthermore, Xue et al. (2018) observed that increasing glutamine in the diet increased performance; however, this effect was not observed in birds challenged with subclinical NE. Xue et al. (2018) also observed fewer jejunal and ileal lesions in challenged birds as a response to glutamine supplementation, as well as an increase in villus to crypt ratio. In the present study, subclinical NE challenge increased the occurrence of jejunal lesions; however, no responses to diet treatments were observed. As glutamine and glutamic acid are measured as the

Table 5. Cecal short-chain fatty acid concentrations (μmol/g) on day 16.

Challenge	Protein	AA	Formic	Acetic	Lactic	Propionic	Butyric	Isobutyric	Valeric	Isovaleric	Succinic	Total SCFA
No	SP	100% AA	1.31	51.11	0.59	2.25	12.19	0.43	0.57	1.21	12.10	28.75
		115% EAA	1.58	68.95	0.89	2.68	14.82	0.42	0.55	4.82	23.00	42.44
		115% AA	1.96	53.39	1.37	2.75	12.62	0.56	0.69	2.53	13.86	33.00
	LP	100% AA	1.40	57.49	1.90	1.81	12.51	0.35	0.46	4.49	20.00	37.55
		115% EAA	0.78	67.93	1.48	2.13	14.99	0.25	0.43	3.48	18.75	40.04
		115% AA	1.68	48.66	0.69	2.07	11.85	0.25	0.41	1.95	16.34	32.87
Yes	SP	100% AA	0.25	45.36	14.25	3.38	33.26	0.84	1.10	2.78	10.68	52.03
		115% EAA	0.45	44.15	17.74	2.72	30.12	0.51	1.12	3.19	8.95	46.52
		115% AA	0.78	49.34	25.55	2.76	29.33	0.76	1.35	2.61	8.45	45.24
	LP	100% AA	0.48	26.90	32.31	1.91	11.74	0.40	0.54	2.86	4.93	22.37
		115% EAA	0.66	40.12	8.16	2.80	18.20	0.70	1.05	3.97	8.25	34.85
		115% AA	0.63	44.12	22.40	2.77	26.59	0.80	1.31	2.18	8.30	41.95
SEM			0.12	2.19	1.87	0.13	1.36	0.29	0.07	0.29	0.97	1.76
Main effects												
Challenge		No	1.45[a]	57.92[a]	1.15[b]	2.28	13.17	0.38[b]	0.52[b]	3.08	17.34[a]	35.77
		Yes	0.54[b]	41.66[b]	20.07[a]	2.72	24.87	0.67[a]	1.08[a]	2.93	8.26[b]	40.49
Protein		SP	1.06	52.05	10.07	2.76	22.06	0.59	0.90	2.86	12.84	41.33
		LP	0.94	47.54	11.16	2.25	15.98	0.46	0.70	3.15	12.76	34.94
AA density		100% AA	0.86	45.21	12.26	2.34	17.43	0.50	0.67	2.84	11.93	35.17
		115% EAA	0.87	55.29	7.07	2.58	19.53	0.47	0.79	3.86	14.74	40.96
		115% AA	1.26	48.88	12.50	2.59	20.10	0.59	0.94	2.31	11.74	38.27
P value												
Challenge			<0.001	<0.001	<0.001	0.104	<0.001	0.001	<0.001	0.199	<0.001	0.153
Protein			0.586	0.246	0.479	0.061	0.004	0.124	0.048	0.886	0.964	0.055
AA			0.223	0.109	0.219	0.680	0.215	0.459	0.214	0.073	0.245	0.361
Challenge × protein			0.326	0.224	0.905	0.857	0.002	0.485	0.986	0.922	0.189	0.012
Challenge × AA			0.622	0.068	0.071	0.901	0.304	0.762	0.291	0.779	0.354	0.371
Protein × AA			0.670	0.930	0.173	0.494	0.131	0.413	0.503	0.236	0.581	0.545
Challenge × protein × AA			0.632	0.372	0.483	0.333	0.121	0.125	0.396	0.177	0.097	0.070

[a,b]Differing superscripts within column group indicate significant differences between means by general linear model with post hoc Tukey test ($P < 0.05$).
Abbreviations: AA, amino acids; EAA, essential amino acids; LP, low protein; SCFA, short-chain fatty acids; SP, standard protein.

glutamic acid in diet AA analysis, therefore, the glutamic acid levels in Supplemental Tables 1 and 2 can be considered representative of dietary glutamine. Comparing SP and LP diets containing 100% AA, glutamic acid was reduced by 30.5%; therefore, LP diets contained less glutamine. Both glutamine and glutamic acid are the primary sources of energy for the GIT enterocytes (Watford et al., 1979), and glutamine has been associated with the mucosal structure and damage repair (Rhoads et al., 1997; Khan et al., 1999). Furthermore, increased dietary threonine and serine have been associated with improved GIT health and function (Faure et al., 2006). The increased SCK activity in challenged birds may be indicative of muscle tissue damage, which was observed as jejunal lesions (Moghadam-Kia et al., 2016). Apparent ileal N digestibility was lower in birds challenged with subclinical NE; however, apparent ileal N digestibility increased in diets with greater AA density. Increasing dietary crystalline AA inclusion levels is known to increase apparent ileal N digestibility as the supplements are highly digestible (Wu, 2013). In the present study, feeding the SP diets which were greater in protein-bound AA and lower in crystalline AA than the LP treatments to challenged birds increased apparent ileal N digestibility. Therefore, feeding higher AA-dense diets including nonessential AA can help GIT recovery and increase apparent ileal N digestibility when under challenge.

Altering the CP of the diet is known to alter the microbiome (Apajalahti and Vienola, 2016) as the bacteria are reliant on substrates entering from the feed consumed. Specialist bacteria located in the hinda, not only is the amount of protein substrate reduced from entering the hindgut, but the entire diet matrix is altered, with greater starch inclusion levels from grains and less soy NSP. Under GIT infection, the microbiome plays a key role in the severity of the infection and recovery time. The NSP-poor conditions created by LP diets reduce beneficial bacteria, encouraging competition from potentially harmful bacteria (Choct et al., 2010). Changes in the cecal SCFA and ileal pH indicate changes in the microbiome due to challenge, resulting in more lactate and butyric acid–producing bacteria. However, reducing CP decreased butyric acid–producing bacteria. Butyric acid is a by-product of bacteria specialized in breaking down NSP such as bifidobacteria and is a primary nutrient for colonocytes with further benefits identified in stimulating host immune defense (Hamer et al., 2010; Sunkara et al., 2011). Therefore, reductions in hindgut butyric acid like that demonstrated in LP diets indicates poor cecal health and prolonging the microbiome recovery. However, increasing the AA density of the diet negated the effects of LP diets in challenged birds on cecal SCFA concentrations, suggesting the changes came from lower inclusions of essential AA rather than CP.

Feeding SP diets and diets with 115% AA that were higher in CP increased growth during the grower period. This greater BWG better prepared birds for NE challenge than LP diets or diets with 100% or 115% EAA. Furthermore, feeding SP diets or diets with 115% AA promoted recovery from NE challenge as seen in the finisher performance. Reducing dietary CP is believed to hinder the proliferation of *C. perfringens*; however, in the present study, *Eimeria spp.* were the predisposing factor for NE. *E. spp.* attack the GIT and release protein into the lumen providing substrates for *C. perfringens* proliferation (Shane et al., 1985), as reflected in the reduced grower apparent ileal N digestibility of challenged birds as N was released into the digesta. Reducing dietary CP does not reduce the severity of NE when coccidiosis is the predisposing factor but does exacerbate the effects as birds are typically lighter and more vulnerable to *C. perfringens* infection.

In conclusion, reducing dietary protein may not reduce the effects of NE, especially when coccidiosis is the predisposing factor. Increased concentrations of AA including nonessential AA in broiler diets can help support GIT development and promote NE infection recovery. Feeding LP diets can theoretically be used to negate NE infection by reducing the substrates available for *C. perfringens;* however, other factors in the diet such as higher Gly availability and decreased NSPs may modulate the microbiome to favor *C. perfringens.* A field study will effectively evaluate the potential benefits of LP diets on negating the occurrence of NE when coccidiosis infection is not present.

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SUPPLEMENTARY DATA

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REFERENCES

Alleman, F., and B. Leclercq. 1997. Effect of dietary protein and environmental temperature on growth performance and water consumption of male broiler chickens. Br. Poult. Sci. 38:607–610.

AOAC 1994. Official Methods of Analysis. Association of Official Analytical Chemists, Washington, D.C.

Apajalahti, J., A. Kettunen, and H. Graham. 2004. Characteristics of the gastrointestinal microbial communities, with special reference to the chicken. Worlds Poult. Sci. J. 60:223–232.

Apajalahti, J., and K. Vienola. 2016. Interaction between chicken intestinal microbiota and protein digestion. Anim. Feed Sci. Technol. 221:323–330.

Aviagen. 2014. Ross 308 Broiler: Nutrition Specifications. Aviagen, Newbridge, Midlothian, Scotland, UK.

Bao, Y. M., and M. Choct. 2010. Dietary NSP nutrition and intestinal immune system for broiler chickens. Worlds Poult. Sci. J. 66:511–518.

Barekatain, R., G. Nattrass, A. J. Tilbrook, K. Chousalkar, and S. Gilani. 2019. Reduced protein diet and amino acid concentration alter intestinal barrier function and performance of broiler chickens with or without synthetic glucocorticoid. Poult. Sci. 98:3662–3675.

Belloir, P., B. Meda, W. Lambert, E. Corrent, H. Juin, M. Lessire, and S. Tesseraud. 2017. Reducing the CP content in broiler feeds: impact on animal performance, meat quality and nitrogen utilization. Animal 11:1881–1889.

Bregendahl, K., J. L. Sell, and D. R. Zimmerman. 2002. Effect of low protein diet on performance and body composition of broiler chicks. Poult. Sci. 81:1156–1167.

Choct, M., Y. Dersjant-Li, J. McLeish, and M. Peisker. 2010. Soy oligosaccharides and soluble non-starch polysaccharides: a review of digestion, nutritive and anti-nutritive effects in pigs and poultry. Asian-Australas J. Anim. Sci. 23:1386–1398.

Cloft, S. E., S. J. Rochell, K. S. Macklin, and W.A. Dozier, III.. 2019. Effects of pre-starter and starter diets varying in amino acid density given to broiler chickens that received coccidiosis vaccination at hatch. Poult. Sci. 98:4878–4888.

Cooper, K. K., G. J. Songer, and F. A. Uzal. 2013. Diagnosing clostridial enteric disease in poultry. J. Vet. Diag. Invest. 25:314–327.

Corzo, A., C. A. Fritts, M. T. Kidd, and B. J. Kerr. 2005. Response of broiler chicks to essential and non-essential amino acid supplementation of low crude protein diets. Anim. Feed Sci. Technol. 118:319–327.

Dahiya, J. P., D. Hoehler, D. C. Wilkie, A. G. Van Kessel, and M. D. Drew. 2005. Dietary glycine concentration affects intestinal *Clostridium perfringens* and *lactobacilli* populations in broiler chickens. Poult. Sci. 84:1875–1885.

Dahiya, J. P., D. C. Wilkie, A. G. Van Kessel, and M. D. Drew. 2006. Potential strategies for controlling necrotic enteritis in broiler chickens in post-antibiotic era. Anim. Feed Sci. Technol. 129:60–88.

Dean, D., T. D. Bidner, and L. L. Southern. 2006. Glycine supplementation to low crude protein, amino acid supplemented diets supports optimal performance of broiler chicks. Poult. Sci. 85:288–296.

Drew, M. D., N. A. Syed, B. G. Goldade, B. Laarveld, and A. G. Van Kessel. 2004. Effects of dietary protein source and level on intestinal populations of *Clostridium perfringens* in broiler chickens. Poult. Sci. 83:414–420.

Evonik Animal Nutrition. 2016. AMINOChick® 2.0. Evonik GMBH, Frankfurt, Germany.

Faure, M., C. Mettraux, D. Moennoz, J. Godin, J. Vuichoud, F. Rochat, D. Breuille, C. Obled, and I. Corthesy-Theulaz. 2006. Specific amino acids increase mucin synthesis and microbiota in dextran sulfate sodium–treated rats. J. Nutr. 136:1558–1564.

Ficken, M. D., and D. P. Wages. 1997. Necrotic enteritis. In: B. W. Calnek (Ed.), Diseases of Poultry. Iowa State Univ. Press, Ames, IA, pp. 261–264.

Hamer, H. M., D. M. A. E. Jonkers, S. A. L. W. Vanhoutvin, F. J. Troost, G. Rijkers, A. de Bru¨ne, A. Bast, K. Venema, and R.-J. M. Brummer. 2010. Effect of butyrate enemas on inflammation and antioxidant status in the colonic mucosa of patients with ulcerative colitis in remission. Clin. Nutr. 29:738–744.

Hilliar, M., N. Huyen, C. K. Girish, R. Barekatain, S. Wu, and R. A. Swick. 2019. Supplementing glycine, serine, and threonine in low protein diets for meat type chickens. Poult. Sci. 98:0–9.

Keerqin, C., S. Wu, B. Svihus, R. A. Swick, N. Morgan, and M. Choct. 2017. An early feeding regime and a high-density amino acid diet on growth performance of broilers under subclinical necrotic enteritis challenge. Anim. Nutr. 3:25–32.

Keyburn, A. L., S. A. Sheedy, M. E. Ford, M. M. Williamson, M. M. Awad, J. I. Rood, and R. J. Moore. 2006. Alpha-toxin of

Clostridium perfringens is not an essential virulence factor in necrotic enteritis in chickens. Infect. Immun. 74:6496.

Khan, J., Y. Iiboshi, L. Cui, M. Wasa, K. Sando, Y. Takagi, and A. Okada. 1999. Alanyl-glutamine-supplemented parenteral nutrition increases luminal mucus gel and decreases permeability in the rat small intestine. J. Parenter. Sci. Technol. 23:24–31.

Kidd, M. T., C. D. McDaniel, S. L. Branton, E. R. Miller, B. B. Boren, and B. I. Fancher. 2004. Increasing amino acid density improves live performance and carcass yields of commercial broilers. J. Appl. Poult. Res. 13:593–604.

Moghadam-Kia, S., C. V. Oddis, and R. Aggarwal. 2016. Approach to asymptomatic creatine kinase elevation. Cleve. Clin. J. Med. 83:37–42.

Morgan, N. K., C. L. Walk, M. R. Bedford, and E. J. Burton. 2014. The effect of dietary calcium inclusion on broiler gastrointestinal pH: Quantification and method optimization. Poult. Sci. 93:354–363.

Murakami, A. E., J. R. G. Franco, E. N. Martins, E. O. Oviedo Rondon, M. I. Sakamoto, and M. S. Pereira. 2003. Effect of electrolyte balance in low-protein diets on broiler performance and tibial dyschondroplasia incidence. J. Appl. Poult. Res. 12:207–216.

Ospina-Rojas, I. C., A. E. Murakami, C. R. Duarte, C. Eyng, C. A. Oliveira, and V. Janeiro. 2014. Valine, isoleucine, arginine and glycine supplementation of low-protein diets for broiler chickens during the starter and grower phases. Br. Poult. Sci. 55:766–773.

Powers, W., and R. Angel. 2008. A review of the capacity for nutritional strategies to address environmental challenges in poultry production. Poult. Sci. 87:1929–1938.

Rhoads, J. M., R. A. Argenzio, W. Chen, R. A. Rippe, J. K. Westwick, A. D. Cox, H. M. Berschneider, and D. A. Brenner. 1997. L-glutamine stimulates intestinal cell proliferation and activates mitogen-activated protein kinases. Am. J. Physiol. 272:G943–G953.

Rochell, S. J., A. Helmbrecht, C. M. Parsons, and R. N. Dilger. 2016. Influence of dietary amino acid reductions and Eimeria acervulina infection on growth performance and intestinal cytokine responses of broilers fed low crude protein diets. Poult. Sci. 95:2602–2614.

Rodgers, N. J., R. A. Swick, M. S. Geier, R. J. Moore, M. Choct, and S.-B. Wu. 2015. A multifactorial analysis of the extent to which eimeria and fishmeal predispose broiler chickens to necrotic enteritis. Avian Dis. 59:38–45.

Rodrigues, I., B. Svihus, M. R. Bedford, R. Gous, and M. Choct. 2018. Intermittent lighting improves resilience of broilers during the peak phase of sub-clinical necrotic enteritis infection. Poult. Sci. 97:438–446.

Shane, S. M., J. E. Gyimah, K. S. Harrington, and T. G. Snider. 1985. Etiology and pathogenesis of necrotic enteritis. Vet. Res. Commun. 9:269–287.

Shimizu, T., K. Ohtani, H. Hirakawa, K. Ohshima, A. Yamashita, T. Shiba, N. Ogasawara, M. Hattori, S. Kuhara, and H. Hayashi. 2002. Complete genome sequence of *Clostridium perfringens*, an anaerobic flesh-eater. Proc. Natl. Acad. Sci. U. S. A. 99:996–1001.

Short, F. J., P. Gorton, J. Wiseman, and K. N. Boorman. 1996. Determination of titanium dioxide added as an inert marker in chicken digestibility studies. Anim. Feed Sci. Technol. 59:215–221.

Sunkara, L. T., M. Achanta, N. B. Schreiber, Y. R. Bommineni, G. Dai, W. Jiang, S. Lamont, H. S. Lillehoj, A. Beker, R. G. Teeter, and G. Zhang. 2011. Butyrate enhances disease resistance of chickens by inducing antimicrobial host defense peptide gene expression. PLoS One 6:e27225.

Vieira, S. L., and C. R. Angel. 2012. Optimizing broiler performance using different amino acid density diets: what are the limits? J. Appl. Poult. Res. 21:149–155.

Wade, B., and A. L. Keyburn. 2015. The True Cost of Necrotic Enteritis. Accessed Sept. 2019. https://www.poultryworld.net/Meat/Articles/2015/10/The-true-cost-of-necrotic-enteritis-2699819W/.

Watford, M., P. Lund, and H. A. Krebs. 1979. Isolation and metabolic characteristics of rat and chicken enterocytes. Biochem. J. 178:589–596.

Wu, G. 2013. Amino Acids: Biochemistry and Nutrition. CRC Press, Florida, USA.

Wu, S., N. Rodgers, and M. Choct. 2010. Optimized necrotic enteritis model producing clinical and subclinical infection of *Clostridium perfringens* in broiler chickens. Avian Dis. 54:1058–1065.

Xue, G. D., R. Barekatain, S. B. Wu, M. Choct, and R. A. Swick. 2018. Dietary L-glutamine supplementation improves growth performance, gut morphology, and serum biochemical indices of broiler chickens during necrotic enteritis challenge. Poult. Sci. 97:1334–1341.