Broilers fed a low protein diet supplemented with synthetic amino acids maintained growth performance and retained intestinal integrity while reducing nitrogen excretion when raised under poor sanitary conditions

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ABSTRACT The present study investigated the effects of supplementing a low protein (**LP**) diet supplemented with key essential amino acids (**AA**) to broilers on growth performance, intestinal tract function, blood metabolites, and nitrogen excretion when the animals were maintained under various sanitary conditions for 35 D after hatching. Three hundred eighty-four one-day-old male broilers (Ross 308) were randomly allotted to groups that received one of 6 dietary treatments in a 2 × 3 factorial arrangement (i.e., 2 environmental conditions and 3 dietary treatments) to give 8 replicates per treatment. Broilers were challenged with 2 environmental conditions (sanitary vs. poor sanitary). The dietary treatments were (1) high protein (**HP**) diet, (2) LP diet, and (3) LP diet with synthetic key essential AA (**LPA**): the LP diet was supplemented with synthetic AA up to the required levels for broilers. On day 14, birds consumed the LP diet impaired growth performance compared with those fed the HP diet, while the average daily weight gain-to-feed conversion ratio of birds fed the LPA diet improved to the level of birds fed the HP diet under poor sanitary conditions ($P < 0.05$). Broilers raised under poor sanitary conditions and fed the LP diet displayed higher ($P < 0.05$) zonula occludens (ZO-1) expression on day 14 than broilers fed either the HP or LPA diet. Under sanitary conditions, birds fed HP and LPA diets showed higher villus height and crypt depth compared with those of broilers fed the LP diet on day 35. Moreover, broilers raised in the poor sanitary environment had higher ($P < 0.05$) serum endotoxins than those raised in the sanitary environment. Broilers fed the LPA diet showed reduced ($P < 0.05$) nitrogen excretion on days 14 and 35 compared with those fed the LP and HP diets independent of the environment. In conclusion, the LPA diet did not impair growth performance under poor sanitary conditions for 14 D after hatch while resulting in lower nitrogen excretion in any environment conditions throughout the experiment.

Key words: broiler, essential amino acid, low protein, nitrogen, tight junction

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INTRODUCTION

The standardized ileal digestibility (**SID**) of the crude protein (**CP**) present in a range of feedstuffs varies between 57 and 90% in broilers (Hoehler et al., 2005). Between 10 and 43% of the undigested protein consumed by broilers is subjected to fermentation by cecal bacteria (Drew et al., 2004; Wilkie et al., 2005), and subsequent production of compounds such as amines, indoles, phenols, cresol, and ammonia can lead to poor gastrointestinal tract (**GIT**) condition and impaired growth performance (Qaisrani et al., 2015a). In addition, Wilkie et al. (2005) reported that diets containing imbalanced levels of amino acids (**AA**) increased the activity of putrefactive bacteria in the broiler gut.

Poor sanitary farm conditions increase pathogenic microorganism activity (*Clostridium perfringens*, *Eimeria spp.*, *Escherichia spp.*, etc.) in broilers (Kaldhusdal, 2000; Wang et al., 2008) and cause chronic immune

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stress and inflammatory responses (Roura et al., 1992). These inflammatory responses increase the nutrient requirements of broilers (Humphrey and Klasing, 2004). In light of this, it is probable that feeding a diet high in CP or with imbalanced AA levels to broilers in poor sanitary conditions could be a cause of reduced production and enteric diseases.

Nitrogen (**N**) originating from the excretion of dietary protein is responsible for high ammonia emission, eutrophication, and soil acidification in intensive livestock production (Belloir et al., 2017). To solve this problem, the use of low protein (**LP**) diets for broilers could be a positive strategy to reduce N excretion (Bregendahl et al., 2002; Angel et al. 2006). Greater use of synthetic AA in LP diets has been indicated as an effective way to reduce N excretion in broilers (Si et al., 2004a). Accordingly, previous studies of Aletor et al. (2000) and Bregendahl et al. (2002) found that a reduction of 1% of CP in the diet decreased N excretion by 10%. However, LP diets need to supply essential AA to maintain the AA requirement of broilers to sustain their growth performance (Belloir et al., 2017).

Nevertheless, inconsistent results regarding the growth performance of broilers fed LP diets have been obtained in various studies because of the different factors such as the level of protein reduction, variation in nutrient specifications, nonessential AA requirements, and differences in acid–base balance (Bregendahl et al., 2002; Ospina-Rojas et al. 2014; Vieira et al., 2016).

To our knowledge, few studies have investigated the effectiveness of feeding broilers an LP diet supplemented with synthetic AA to levels that fulfill the SID AA requirement while the animals are housed under poor sanitary conditions.

Therefore, the present study was designed to observe the growth performance, aspects of GIT structure and function, serum endotoxin levels, and N excretion of broilers fed an LP diet supplemented with synthetic AA and raised in either sanitary or poor sanitary environment for 35 D after hatching. The hypothesis tested was that broilers fed an LP diet supplemented with synthetic AA to levels that provided the ideal AA ratio would maintain growth performance and GIT integrity while reducing N excretion when kept in poor sanitary conditions.

MATERIALS AND METHODS

The experimental protocol for the present study was reviewed and approved by the Animal Ethics Committee of the Chungnam National University (Protocol No. CNU-00963). Birds were managed and cared in accordance with the guidelines of the Ross Broiler Management Handbook (Avigen, 2014a).

Birds and Experimental Design

A total of 384, 1-day-old (Ross 308) male broilers with initial body weight of 43.5 ± 0.20 g (mean ± standard error of mean) were randomly allocated to one of six treatments in a 2 × 3 (2 environment conditions and 3 dietary treatments) factorial arrangement to give 8 replicates per treatment (8 birds per pen). Birds were stratified into 2 groups of difference environmental conditions, sanitary (previously unpopulated, disinfected and clean) and poor sanitary (previously populated, nondisinfected, and uncleaned) environmental conditions (Pastorelli et al., 2012; Shin et al., 2017), with 3 dietary treatments for each. Three dietary treatments were (1) HP diet: a diet with 23.5 and 20.5% CP in starter and finisher phases, respectively; (2) LP diet: a diet with 18 and 17% CP in starter and finisher phases, respectively; and (3) LP diet with synthetic AA (LPA): the LP diet was supplemented with essential AA (Val, Ile, Arg, Trp, Gly, and Ser) together with Met, Lys, and Thr to fulfill the ideal AA requirement (Hoehler et al., 2005) of broilers.

Broilers were placed on wire-floor pens (0.85 m × 0.55 m × 0.35 m), and each pen was equipped with 2 nipple drinkers and a metal trough. Temperature of the research facility strictly followed the Ross 308 bird management guidelines (2014). Birds were offered the experimental diets on an *ad libitum* basis for 35 days. All birds had free access to fresh clean drinking water via nipple drinkers throughout the experiment period.

Experimental Diets

Diets were formulated based on corn–soybean meal and wheat to meet or exceed the nutrient recommendations of Ross broiler 308 specification (Avigen, 2014b). Furthermore, AA requirements of the HP and LPA diets were followed the SID AA recommendations suggested by Hoehler et al. (2005) (Tables 1 and 2). Experimental diets did not include any feed additives. All the diets were produced in mash form.

Growth Performance

Pen basis initial body weights were measured and recorded on arrival. Thereafter, body weights were measured at the end of every week until day 35 of the experiment. Average daily gain (**ADG**) was calculated using body weights data. Feed intake on weekly basis was measured based on feed disappearance in each cage. Finally, mortality-corrected average daily feed intake (**ADFI**) and feed conversion ratio (**FCR**) of each cage were calculated in each respective week of the experiment.

Sample Collection

Sample collections occurred on days 14 and 35. One broiler (that weighed close to the mean body weight) was selected from all cages at a time for sample collection. Before euthanasia of the selected birds, blood samples were collected into 4 Vacutainer tubes (4 mL for each). Two Vacutainers contained spray-coated silica and a polymer gel for serum separation (BD Vacutainer; SST II advance, Plymouth, PL6 7BP, UK) and the other

Table 1. Composition (%, as-fed basis) of the experimental diets.

	Starter (day 0 to 14)			Grower-finisher (day 15 to 35)		
Item	HP[1]	LP[2]	LPA[3]	HP	LP	LPA
Maize 8%	55.30	73.97	71.71	55.30	56.85	55.86
Wheat 11%	-	-	-	8.49	21.33	21.33
Soybean meal 47%	34.15	18.03	18.03	24.56	13.12	13.12
Fish meal 65%	4.23	3.00	3.00	5.00	4.00	4.00
Soy oil	3.19	1.30	1.30	3.67	2.73	2.73
Salt	0.37	0.41	0.41	0.31	0.33	0.33
Dicalcium phosphate	1.36	1.62	1.62	1.11	1.29	1.29
Limestone	0.66	0.73	0.73	0.56	0.61	0.61
Mineral/vitamin premix[4]	0.30	0.30	0.30	0.30	0.30	0.30
Lysine-HCl	0.07	0.64	0.64	0.14	0.55	0.55
DL-Methionine	0.25	-	0.41	0.23	-	0.35
L-Threonine	-	-	0.26	0.04	-	0.23
L-Tryptophan	-	-	0.04	-	-	0.29
L-Valine	-	-	0.32	0.04	-	0.26
L-Arginine	-	-	0.38	-	-	0.34
L-Glycine	0.12	-	0.39	0.22		0.41
L-Serine	-	-	0.15	-	-	-
L-Isoleucine	-	-	0.31	0.04	-	0.28

[1]High protein diet.
[2]Low protein diet.
[3]Lower protein diet with synthetic amino acids.
[4]Supplied per kilogram of total diets: Fe ($FeSO_4.H_2O$), 80 mg; Zn ($ZnSO_4 \cdot H_2O$), 80 mg; Mn ($MnSO_4 \cdot H_2O$) 80 mg; Co ($CoSO_4 \cdot H_2O$) 0.5 mg; Cu ($CuSO_4 \cdot H_2O$) 10 mg; Se (Na_2SeO_3) 0.2 mg; I, ($Ca(IO_3) \cdot 2H_2O$) 0.9 mg; vitamin A, 24,000 IU; vitamin D_3, 6,000 IU; vitamin E, 30 IU; vitamin K, 4 mg; thiamin, 4 mg; riboflavin, 12 mg; pyridoxine, 4 mg; folacine, 2 mg; biotin, 0.03 mg; vitamin B_8, 0.06 mg; niacin, 90 mg; pantothenic acid, 30 mg.

two contained spray-coated K2 EDTA for anticoagulant (BD Vacutainer, Franklin Lakes, NJ). Collected blood samples were quickly transferred to a laboratory for serum and plasma separation.

Afterward, selected broilers were euthanized by cervical dislocation, abdominal incisions were made, and the ileum was separated from the GIT. The ileum was defined as the segment of the small intestine that extended from Meckel's diverticulum to the ileocecal junction. A 3-cm piece of the ileum was removed and flushed with ice-cold phosphate-buffered saline (**PBS**) at pH 7.4. The samples were placed into plastic containers containing 10% formaldehyde for fixation and stored until further processing for analysis. Furthermore, another ileal sample was excised and mucosal scrapings were collected. Scraped mucosa were stored in the microtube containing RNAiso Plus (Takara Bio Inc., Kusatsu, Japan) solution and quickly stored at −80°C.

Sample Preparation and Laboratory Analyses

Blood samples were centrifuged (1248R; LaboGene, Denmark) at 3,000 × *g* for 10 min at 4°C for separation of plasma and serum. Blood urea nitrogen (**BUN**) levels were quantified by the urease enzymatic kinetic method (Morishita et al., 1997).

Serum endotoxin levels were tested by using a limulus amebocyte lysate (LAL) chromogenic endotoxin quantitation kit (Thermo Scientific Pierce, Rockford, IL), in accordance with the method described by Laugerette et al. (2015). In brief, serum samples were diluted and heated at 70°C for 15 min. Afterward, LAL and chromogenic substrate were added into heated blood, and then 25% of acetic acid was added to end the reaction. Sample absorbance was then measured at 410 nm using a microplate spectrophotometer (Epoch; Bio-Tek, Winooski, VT). The endotoxin levels were calculated using a preestablished standard curve (Laugerette et al., 2015).

Ileal tissue samples were taken and dehydrated for 16 h, followed by impregnation in paraffin wax. Six transverse sections with 4 to 6 μm of thickness were cut by consuming a microtome machine (Leica RM 2155; Leica Biosystems GmbH, Germany) and placed on glass slides. Subsequently, glass slides that contained ileal sections were heated at 57°C until dry. Afterward, dried slides were stained with hematoxylin and eosin stain besides roofed by another slide. Morphological indices were measured using the NIS-Elements Viewer software (version 4.20; NIS-Elements, Nikon) together with an inverted microscope (Eclipse TE2000; Nikon Instruments Inc., Melville, NY) that had a calibrated eyepiece graticule. Ten average-sized and well-oriented villi and their associated crypts were used to measure villus height (starting from crypt mouth to the villus tip) and crypt depth (base region of transition among the crypt and villi) (Law et al., 2018).

Total RNA was extracted from ileum tissues using RNAiso plus (Takara Bio Inc., Kusatsu, Japan). The RNA concentration was confirmed by BioSpec-nano (Shimadzu, Kyoto, Japan). After 500 ng of RNA was heated at 65°C for 5 min, cDNA was synthesized using ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan) following Elgawish et al. (2018). The relative gene expressions of zona occludin 1 (**ZO 1**), occludin 1 (**OCLN 1**), and claudin 1 (**CLDN1**) were confirmed

Table 2. Calculated and analyzed nutritional compositions of each diet.

Item	Starter (day 0 to 14)			Grower-finisher (day 15 to 35)		
	HP[1]	LP[2]	LPA[3]	HP	LPA	LP
Energy (Kcal/kg)	3,040	3,040	3,040	3,138	3,138	3,138
Dry matter (%)	88.43	87.74	87.68	88.42	88.34	88.27
Crude protein (%)	23.50	18.00	18.00	20.50	17.00	17.00
SID[4] Lysine (%)	1.25	1.25	1.25	1.11	1.11	1.11
SID methionine (%)	0.60	0.27	0.67	0.55	0.26	0.60
SID cystine (%)	0.31	0.24	0.24	0.28	0.23	0.23
SID M + C (%)	0.91	0.51	0.91	0.83	0.49	0.83
SID threonine (%)	0.80	0.70	0.80	0.72	0.66	0.72
SID tryptophan (%)	0.25	0.16	0.20	0.21	0.15	0.18
SID isoleucine (%)	0.90	0.60	0.85	0.79	0.55	0.78
SID arginine (%)	1.41	0.92	1.29	1.17	0.84	1.17
SID valine	0.99	0.69	0.99	0.89	0.64	0.89
SID glycine (%)	1.00	0.62	1.00	1.00	0.60	1.00
SID serine (%)	1.02	0.74	0.88	0.88	0.69	0.68
SID Gly + Ser (%)	2.02	1.36	1.88	1.88	1.29	1.68
Analyzed chemical composition[5]						
Crude protein (%)	23.34	17.82	18.07	19.88	16.84	17.11
Total lysine (%)	1.36	1.37	1.47	1.25	1.34	1.20
Total methionine (%)	0.69	0.40	0.68	0.57	0.37	0.62
Total cystine (%)	0.41	0.36	0.34	0.40	0.32	0.33
Total M + C	1.10	0.76	1.02	0.97	0.69	0.95
Total threonine (%)	0.83	0.87	0.93	0.80	0.72	0.82
Total tryptophan (%)	0.28	0.21	0.25	0.27	0.16	0.18
Total isoleucine (%)	0.93	0.64	0.90	0.83	0.56	0.74
Total arginine (%)	1.45	1.09	1.38	1.23	0.99	1.27
Total valine (%)	0.96	0.74	0.94	0.84	0.68	0.81
Total glycine (%)	1.19	0.91	1.05	1.21	0.81	1.11
Total serine (%)	1.07	0.84	0.85	1.03	0.75	0.79
Total Gly + Ser (%)	2.26	1.75	1.90	2.24	1.56	1.90

[1]High protein diet.
[2]Low protein diet.
[3]Lower protein diet with synthetic amino acids.
[4]Standardized ileal digestibility.
[5]% as-fed basis.

by quantitative real-time PCR (Step One Plus real-time system; Thermo Fisher Scientific) with SYBR green PCR reagent (TOPreal qPCR 2X Premix; Enzynomics, Daejeon, Korea) and normalized to the level of beta-actin as a reference gene. The primer sequences for those genes are shown in Table 3.

Average nitrogen excretion was determined using pre-derived equations according to Belloir et al. (2017). Whole-body nitrogen content in broilers was assumed as a constant (29 g/kg) according to Bregendahl et al. (2002).

$$\text{Nitrogen intake}(g) = \frac{\text{Feed intake}(g) \times \text{Crude protein in the diet}(\%)}{6.25}$$

$$\text{Nitrogen retention}(g) = \text{Whole body nitrogen}\left(\frac{g}{kg}\right) \times \frac{\text{Body weight gain}(g)}{1,000}$$

$$\text{Nitrogen Excretion}(g) = \text{Nitrogen intake}(g) - \text{Nitrogen retention}(g)$$

Statistical Analyses

Data were analyzed as a 2 × 3 factorial arrangement using the SPSS software (version 21; IBM SPSS 2012). The general linear model procedure of 2-way analysis of variance was performed to determine the main effects of dietary treatments, environmental conditions, and their interactions. Pen was used as the experimental unit for growth performance and N excretion. Selected pen-based individual broilers were considered as experimental units for all other measures including ileal morphology, tight junction gene expressions, and blood metabolites. Mean differences observed in the treatment were considered significant at $P < 0.05$. When treatment effects were significant ($P < 0.05$), means were separated using the Tukey multiple comparison test procedures.

RESULTS

All birds remained healthy and performed well; mortality was 1.82% during the 35 D of the experimental period.

The effect of an LP diet on the growth performance of broilers under 2 environmental conditions is presented in Table 4. An interaction ($P < 0.01$) between diet and environmental condition ($P < 0.05$) was found for

Table 3. The primers used for quantitative real-time PCR.

Gene		Primer sequence	Gene Bank accession
ZO1[1]	Forward	5′-GCTCACAAGCTACGCAAAAA-3′	XM 015278981.1
	Reverse	5′-ACCATCTGCCTTTCCTTCAG-3′	
OCLN1[2]	Forward	5′-TCTGCCTCATCTGCTTCTTC-3′	NM 205128.1
	Reverse	5′-TTCTTCACCCACTCCTCCA-3′	
CLDN1[3]	Forward	5′-TGTCATCTTCAGCACCTTCC-3′	NM 001013611.2
	Reverse	5′-CAGCGCATGCATACAGTTAC-3′	
ß-actin	Forward	5′-CCAAAGCCAACAGAGAGAAG-3′	NM 205518.1

[1]Zonula occludens-1.
[2]Occludin-1.
[3]Claudin-1.

ADG and FCR from the starter period to day 14. In both environmental conditions, broilers fed an LP diet had reduced ADG and increased FCR compared with broilers fed an HP diet. However, under the poor sanitary conditions, broilers fed the LPA diet showed improved performance (ADG and FCR) to the level of that achieved by the broilers fed the HP diet. In contrast, under sanitary conditions, broilers fed diet LPA showed impaired growth performance compared with those fed diet HP. On the other hand, the LP diet group showed poorer ($P < 0.01$) ADG and FCR than the HP group, while the LPA group showed ADG and FCR values similar to those of the HP group for the grower-finisher period (i.e., days 15 to 35), independent of environmental conditions. For the overall experiment period, broilers fed the LPA diet showed ADG and FCR values similar to those of broilers fed the HP diet independent of the environmental conditions.

Environmental conditions and dietary treatments showed no interaction effect on feed intake during the 35-D research period. However, broilers fed LP and LPA diets showed reduced ($P < 0.01$) ADFI compared with those fed an HP diet from hatching to 35 D after hatching.

The effects of dietary treatments and environment on intestinal architecture are presented in Table 5. No interaction was observed between diet and environmental conditions on intestinal architecture on day 14. Housing broilers in poor sanitary conditions decreased ($P < 0.05$) villus height (**VH**), crypt depth (**CD**), and the VH:CD on day 14 compared with broilers housed in sanitary conditions. However, no diet-directed effect ($P > 0.05$) was observed on intestinal architecture on day 14. On day 35, there was an interaction ($P < 0.05$) between diet and environmental conditions on VH and CD. Broilers fed HP and LPA diet showed higher ($P < 0.05$) VH and CD when housed in sanitary condition than birds in poor sanitary conditions.

The effects of feeding different diets on blood metabolites under different environmental conditions are

Table 4. Effect of a low protein diet supplemented with synthetic amino acids on growth performance of broilers housed in 2 different environmental conditions from hatch to 35 D.[1]

		Average daily gain, g			Average daily feed intake, (g)			Feed conversion ratio, (g/g)		
Dietary treatments		Day 1 to 14	Day 15 to 35	Day 1 to 35	Day 1 to 14	Day 15 to 35	Day 1 to 35	Day 1 to 14	Day 15 to 35	Day 1 to 35
Sanitary[2]	HP	33.0^{a}	78.3	60.2	39.3	126.7	91.7	1.191^{c}	1.617	1.523
	LP	21.1^{c}	60.4	44.7	34.2	113.2	81.6	1.638^{a}	1.876	1.831
	LPA	27.3^{b}	77.0	57.1	36.3	120.4	86.8	1.331^{b}	1.570	1.521
Poor sanitary[3]	HP	27.4^{b}	78.9	58.3	36.8	124.9	89.6	1.348^{b}	1.586	1.541
	LP	21.0^{c}	60.5	44.7	33.8	113.1	81.4	1.610^{a}	1.871	1.821
	LPA	25.1^{b}	74.5	54.7	35.5	115.0	83.2	1.418^{b}	1.549	1.525
SEM[4]		0.593	1.734	1.173	0.847	2.257	1.440	0.032	0.029	0.024
Main effect means										
Environment	Sanitary	27.1	71.9	54.0	36.6	120.1	86.7	1.39	1.69	1.63
	Poor sanitary	24.5	71.2	52.6	35.4	117.7	86.7	1.46	1.67	1.63
Diets	HP[5]	30.2	78.6^{a}	59.2^{a}	38.0^{a}	125.8^{a}	90.7^{a}	1.27	1.60^{b}	1.53^{b}
	LP[6]	21.1	60.5^{b}	44.7^{c}	34.0^{b}	113.1^{b}	81.5^{b}	1.62	1.87^{a}	1.83^{a}
	LPA[7]	26.2	75.8^{a}	55.9^{b}	35.9^{b}	117.7^{b}	85.0^{b}	1.37	1.56^{b}	1.52^{b}
P values	Environment[8]	0.001	0.635	0.136	0.075	0.198	0.104	0.009	0.421	0.836
	Diet[9]	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
	Interaction[10]	0.001	0.639	0.542	0.440	0.486	0.510	0.021	0.901	0.842

[a,b,c]Values in a row with different superscripts differ significantly ($P < 0.05$).
[1]Values are the mean of 8 replicates per treatment.
[2]Sanitary: previously unpopulated, disinfected, and clean.
[3]Poor sanitary: previously populated, nondisinfected, and uncleaned.
[4]Pooled standard error of mean.
[5]High protein diet: a diet with 23.5 and 20.5% crude protein (**CP**) in starter and finisher phases, respectively.
[6]Low protein diet: a diet with 18 and 17% CP in starter and finisher phases, respectively.
[7]LP with synthetic amino acids (**LPA**): the LP diet was supplemented with key essential amino acids.
[8]Environmental condition (sanitary vs. poor sanitary).
[9]Dietary treatment.
[10]Interaction between dietary treatment and environmental condition.

Table 5. Effect of a low protein diet supplemented with synthetic amino acids on intestinal architecture of broilers housed in 2 different environmental conditions from hatch to 35 D.[1]

Dietary treatments		Villus height, (μm)		Crypt depth, (μm)		Villus height: crypt depth ratio	
		Day 14	Day 35	Day 14	Day 35	Day 14	Day 35
Sanitary[2]	HP	474	732[a]	57	104[a]	9.9	7.5
	LP	487	448[b]	61	68[b]	9.8	6.9
	LPA	455	796[a]	63	116[a]	8.7	7.5
Poor sanitary[3]	HP	361	401[b]	54	67[b]	7.5	6.6
	LP	339	363[b]	51	59[b]	7.4	7.4
	LPA	325	403[b]	57	63[b]	6.4	7.3
SEM[4]		28.277	54.331	3.858	6.932	0.834	0.537
Main effect means							
Environment	Sanitary	472	659	60	96	9.5	7.3
	Poor sanitary	342	389	54	63	7.1	7.1
Diets	HP[5]	418	566	55	86	8.7	7.0
	LP[6]	414	406	56	64	8.6	7.2
	LPA[7]	390	600	60	90	7.6	7.4
P values	Environment[8]	0.001	0.001	0.043	0.001	0.001	0.605
	Diet[9]	0.580	0.002	0.350	0.001	0.353	0.738
	Interaction[10]	0.830	0.017	0.701	0.009	0.997	0.456

[a,b]Values in a row with different superscripts differ significantly ($P < 0.05$).
[1]Values are the mean of 8 replicates per treatment.
[2]Sanitary: previously unpopulated, disinfected and clean.
[3]Poor sanitary: previously populated, nondisinfected, and uncleaned.
[4]Pooled standard error of mean.
[5]High protein diet: a diet with 23.5 and 20.5% crude protein (**CP**) in starter and finisher phases, respectively.
[6]Low protein diet: a diet with 18 and 17% CP in starter and finisher phases, respectively.
[7]LP with synthetic amino acids (**LPA**): the LP diet was supplemented with key essential amino acids.
[8]Environmental condition (sanitary vs. poor sanitary).
[9]Dietary treatment.
[10]Interaction between dietary treatment and environmental condition.

presented in Table 6. There was no interaction effect ($P > 0.05$) on serum endotoxin levels on days 14 or 35. However, broilers housed in poor sanitary conditions showed higher ($P < 0.01$) endotoxin levels than broilers housed in sanitary conditions on days 14 and 35. On day 14, broilers fed the LP diet showed increased ($P < 0.01$) plasma urea nitrogen levels compared with broilers fed the HP and LPA diets when they were housed under sanitary environmental conditions.

The effects of feeding on ileal tight junction gene expression of broilers fed different diets under different environmental conditions are presented in Table 7. There was a significant interaction of diet and environment on the expression of ZO 1 on day 14; broilers fed the LP diet under poor sanitary conditions showed increased ($P < 0.01$) expression of ZO 1 compared with broilers fed the LPA and HP diets. In addition, broilers fed the LP diet showed increased ($P < 0.05$) claudin 1 and ZO 1 expression on day 35 compared with broilers fed the HP diet, and this was independent of the environmental conditions.

The calculated values for N excretion by birds subjected to different dietary treatments in the 2 environmental conditions are shown in Table 8. There was no interaction of diet and environment on N excretion on days 14 or 35. Broilers fed the LPA diet showed decreased ($P < 0.01$) N excretion compared with those fed the LP and HP diets, while broilers fed the LP diet showed lower N excretion on days 14 and 35 than broilers fed the HP diet. Broilers housed in poor sanitary conditions showed higher ($P < 0.05$) N excretion at 14 D after hatching than broilers housed in a sanitary environment.

DISCUSSION

This study examined the hypothesis that broilers kept in poor sanitary conditions and fed an LP diet supplemented with synthetic AA to fulfill the birds' AA requirement would maintain growth performance and GIT integrity while reducing nitrogen excretion. It was presumed that lowering the protein content in a diet provides fewer substrates from which putrefactive bacteria in the distal end of the GIT can produce toxic compounds (i.e., ammonia, amines, indoles, phenols) that could be a cause of disturbance of the large intestine and impaired growth (Kaldhusdal, 2000; Qaisrani et al., 2015b).

The results of the present study revealed that broilers under poor sanitary conditions and fed LPA diet showed ADG and FCR values that were similar to those of broilers fed an HP diet in same conditions on day 14. In addition, broilers fed LPA diet under poor sanitary conditions resulted 16.12% lower blood urea nitrogen level than broilers fed HP diet in poor sanitary conditions. Therefore, we can assume that AA catabolism is higher in broilers fed HP diet under poor sanitary conditions which is consider as lost to the broilers. Moreover, HP diet was formulated with higher inclusion level of soybean meal than LPA diet (341.5 g/kg vs. 180.3 g/kg). Intensive use of soybean leads to an influential threat to the biodiversity of the world (Fearnside, 2001). Moreover, N excretion results of this study confirmed the low efficiency of N utilization in HP diet under either environmental condition. Consequently, we recommended LPA diet is more suitable for the poor sanitary conditions because it can achieve the

Table 6. Effect of a low protein diet supplemented with synthetic amino acids on blood metabolites of broilers housed in 2 different environmental conditions from hatch to 35 D.[1]

Dietary treatments		Serum endotoxins, EU/mL		Plasma urea nitrogen, mg/dL	
		Day 14	Day 35	Day 14	Day 35
Sanitary[2]	HP	40.3	40.0	1.13[b]	0.58
	LP	35.0	33.1	1.76[a]	0.45
	LPA	35.1	39.4	0.88[cb]	0.42
Poor sanitary[3]	HP	61.3	61.8	0.62[cb]	0.44
	LP	66.8	57.1	0.61[cb]	0.61
	LPA	47.4	64.7	0.52[c]	0.52
SEM[4]		8.009	6.662	0.136	0.072
Main effect means					
Environment	Sanitary	36.8	37.4	1.26	0.48
	Poor sanitary	58.5	61.2	0.58	0.52
Diets	HP[5]	50.8	50.7	0.88	0.51
	LP[6]	50.9	45.1	1.18	0.53
	LPA[7]	41.3	52.1	0.70	0.47
P values	Environment[8]	0.006	0.001	0.001	0.473
	Diet[9]	0.412	0.557	0.004	0.730
	Interaction[10]	0.495	0.973	0.013	0.089

[a,b]Values in a row with different superscripts differ significantly ($P < 0.05$).
[1]Values are the mean of 8 replicates per treatment.
[2]Sanitary: previously unpopulated, disinfected and clean.
[3]Poor sanitary: previously populated, nondisinfected, and uncleaned.
[4]Pooled standard error of mean.
[5]High protein diet: a diet with 23.5 and 20.5% crude protein (**CP**) in starter and finisher phases, respectively.
[7]Low protein diet: a diet with 18 and 17% CP in starter and finisher phases, respectively.
[6]LP with synthetic amino acids (**LPA**): the LP diet was supplemented with key essential amino acids.
[8]Environmental condition (sanitary vs. poor sanitary).
[9]Dietary treatment.
[10]Interaction between dietary treatment and environmental condition.

similar growth performance that is obtained from those fed HP diet for the starter phase. Nevertheless, comparable growth performance was found for broilers fed the LPA and HP diets regardless of the environmental conditions for the grower-finisher phase. Interestingly, Ospina-Rojas et al. (2014) reported that the CP level could be reduced by 3% when the diet was supplemented with synthetic AA without any adverse effects on the

Table 7. Effect of a low protein diet supplemented with synthetic amino acids on tight junction gene expression in the ileal mucosa of broilers housed in 2 different environmental conditions from hatch to 35 D.[1]

Dietary treatments		Claudin 1		ZO 1		Occludin 1	
		Day 14	Day 35	Day 14	Day 35	Day 14	Day 35
Sanitary[2]	HP	1.77	1.36	1.24[c]	0.73	0.92	1.01
	LP	2.25	2.53	2.84[c]	2.97	1.29	1.44
	LPA	1.32	1.79	1.92[c]	1.34	1.58	1.58
Poor sanitary[3]	HP	1.42	1.86	2.92[c]	1.08	1.88	1.11
	LP	3.92	4.52	13.40[a]	1.81	2.80	1.41
	LPA	3.45	2.02	9.07[b]	1.59	4.08	1.07
SEM[4]		0.787	0.639	0.705	0.441	0.359	0.249
Main effect means							
Environment	Sanitary	1.78	1.90	2.00	1.68	1.27	1.34
	Poor sanitary	2.92	2.80	8.46	1.49	2.92	1.20
Diets	HP[5]	1.59	1.61[b]	2.08	0.91[b]	1.40[b]	1.06
	LP[6]	3.08	3.53[a]	8.11	2.39[a]	2.83[a]	1.43
	LPA[7]	2.38	1.91[ab]	5.49	1.47[ab]	2.05[ab]	1.32
P values	Environment[8]	0.090	0.101	0.001	0.607	0.001	0.475
	Diet[9]	0.195	0.016	0.001	0.012	0.003	0.334
	Interaction[10]	0.270	0.357	0.001	0.186	0.125	0.444

[a,b]Values in a row with different superscripts differ significantly ($P < 0.05$).
[1]Values are the mean of 8 replicates per treatment.
[2]Sanitary: previously unpopulated, disinfected and clean.
[3]Poor sanitary: previously populated, nondisinfected, and uncleaned.
[4]Pooled standard error of mean.
[5]High protein diet: a diet with 23.5 and 20.5% crude protein (**CP**) in starter and finisher phases, respectively.
[6]Low protein diet: a diet with 18 and 17% CP in starter and finisher phases, respectively.
[7]LP with synthetic amino acids (**LPA**): the LP diet was supplemented with key essential amino acids.
[8]Environmental condition (sanitary vs. poor sanitary).
[9]Dietary treatment.
[10]Interaction between dietary treatment and environmental condition.

Table 8. Effect of a low protein diet supplemented with synthetic amino acids on nitrogen utilization of broilers housed in 2 different environmental conditions from hatch to 35 D.[1]

Dietary treatments		Nitrogen excretion, (g)	
		Day 1 to 14	Day 15 to 35
Sanitary[2]	HP	7.3	39.5
	LP	5.3	27.8
	LPA	3.6	21.9
Poor sanitary[3]	HP	8.3	38.0
	LP	5.1	20.3
	LPA	4.1	27.8
SEM[4]		0.276	0.982
Main effect means			
Environment	Sanitary	5.4	29.7
	Poor sanitary	5.8	28.7
Diets	HP[5]	7.8[a]	38.8[a]
	LP[6]	5.2[b]	27.8[b]
	LPA[7]	3.9[c]	21.1[c]
P values	Environment[8]	0.049	0.204
	Diet[9]	0.001	0.001
	Interaction[10]	0.119	0.692

[a,b,c]Values in a row with different superscripts differ significantly ($P < 0.05$).
[1]Values are the mean of 8 replicates per treatment.
[2]Sanitary: previously unpopulated, disinfected and clean.
[3]Poor sanitary: previously populated, nondisinfected, and uncleaned.
[4]Pooled standard error of mean.
[5]High protein diet: a diet with 23.5 and 20.5% crude protein (**CP**) in starter and finisher phases, respectively.
[6]Low protein diet: a diet with 18 and 17% CP in starter and finisher phases, respectively.
[7]LP with synthetic amino acids (**LPA**): the LP diet was supplemented with key essential amino acids.
[8]Environmental condition (sanitary vs. poor-sanitary).
[9]Dietary treatment.
[10]Interaction between dietary treatment and environmental condition.

performance of broiler chickens on day 42, in agreement with the results of the present study for the grower-finisher phase. Furthermore, broilers at the grower phase showed reduced requirement of nonessential AA that explains the equal growth performance results obtained for broilers fed the HP and LPA diets in the present study (Kamran et al., 2011).

Unexpectedly, we found reduced feed intake in broilers fed an LPA diet; however, Carew et al. (1998) and Si et al. (2004b) demonstrated that feeding a diet to which crystalline AA were added reduced the feed consumption of broilers (in the present study, 29 g/kg and 27.1 g/kg of AA in synthetic form were added on a weight basis to the starter and grower-finisher diets, respectively). Some prior studies (Kumta and Harper, 1962; Peng and Harper, 1970) suggested an aminostatic hypothesis, namely, that AA levels or fluctuation patterns in plasma may serve as a signal to an appetite-controlling mechanism. With this in mind, the higher rate of absorption of the synthetic AA in the LPA diet could cause a sudden influx of free AA in plasma that might affect feed intake. On the other hand, Si et al. (2004b) suggested that the level of Trp in the diet may also affect the feed intake of broilers. Tryptophan acts as a precursor to serotonin, a neurotransmitter that is involved in stimulating feed intake (Si et al., 2004b). This perspective could explain the low feed intake in broilers fed an LPA diet considering that the starter and grower-finisher diets contained 36 and 29% less Trp, respectively, than the HP diet.

The results of the present study showed that broilers reared in poor sanitary conditions showed reduced VH, CD, and VH:CD on day 14 compared with broilers in sanitary conditions. The study by Ao et al. (2012) showed that higher pathogenic bacterial load in the GIT reduced the VH and VH:CD. Higher pathogenic bacterial load was evidenced by the elevated serum endotoxin levels found in broilers housed in poor sanitary conditions in this study. Moreover, under these conditions, the birds may have used extra energy to maintain GIT function owing to immune challenges, instead of maintaining gut morphology. The present study did not find any diet effect on VH and CD under the poor sanitary conditions. In addition, broilers exposed to the sanitary conditions and fed either LPA or HP diet showed higher VH and CD compared with broilers fed LP diet on day 35. Law et al. (2018) found that broilers fed LP diets (i.e., 17.2 and 15.6% CP in the starter and finisher diets, respectively) supplemented with synthetic AA displayed poor intestinal architecture compared with broilers fed HP diets (21 and 19% CP in in starter and finisher diets, respectively) on day 35. Prior studies (Lensing et al., 2007; Law et al., 2018) suggested that the adverse effect of an LP diet on intestinal architecture could be attributed to a reduction in the levels of nonessential AA such as glycine, glutamine, and proline, which are necessary for the development of the gut epithelium and for the production of digestive secretions and mucin. The present study demonstrated that feeding an LP diet supplemented with key essential AA together with glycine had no adverse effects on the VH of broilers when they were housed in sanitary conditions.

Endotoxin levels in the blood reflect intestinal barrier integrity and function (Li et al., 2018). Wang et al. (2003) reported that broilers maintained in a poor sanitary environment showed increased plasma levels of endotoxin compared with broilers housed under sanitary conditions, consistent with the results of the present study (Table 6). Endotoxins are lipopolysaccharides that are present in the outer membrane of gram-negative bacteria (Raetz and Whitfield, 2002). High populations of *C. perfringens* and *Escherichia* elevate blood endotoxin levels in broilers both directly and indirectly (Li et al., 2018). *C. perfringens* is known as the causative agent of necrotic enteritis; it leads to mucosal atrophy that compromises epithelial permeability (Timbermont et al., 2011; Li et al., 2018).

BUN indicates the efficiency of AA utilization in broiler diets. Ospina-Rojas et al. (2014) reported that broilers fed an LP diet (19% CP) supplemented with synthetic AA (Lys, Met, Thr) had reduced uric acid levels compared with broilers fed a high protein diet (22% CP) during the starter period. Donsbough et al. (2010) reported that both blood uric acid and urea nitrogen can be used as indicators of AA consumption in broilers. The reduced BUN levels in broilers fed an LPA diet compared with broilers fed either an HP or

an LP diet likely indicate that the AA balance in the LPA diet was much closer to the broilers' requirements compared with the HP and LP diets. Moreover, broilers fed LPA diet containing high level of unbound AA (29 g/kg and 27.1 g/kg in starter and grower-finisher diets, respectively). Interestingly, it was confirmed that crystalline AA absorb more rapidly than protein-bound AA. In this scenario, Wu (2009) reported that diverse rates of AA absorbance reasoning an elevated AA catabolism to response imbalance AA levels in systemic circulation. In contrast, broilers fed higher crystalline AA level did not show elevated BUN levels in either environment condition in the present study. Therefore, we can assume that the inclusion levels of crystalline AA to protein-bound AA ratio did not make any AA imbalance in the present study.

Furthermore, broilers fed the LPA diet showed lower N excretion than broilers fed the LP diet, suggesting that nitrogen utilization was more efficient in the animals on the LPA diet. Broilers fed an LPA diet reduced N excretion by 50.5 and 45.4% compared with broilers fed an HP diet in the starter and grower-finisher phases, respectively. These results agree with those of Bregendahl et al. (2002), who concluded that reducing the CP level by 1% could result in a 10% reduction in N excretion. In support of this conclusion, the LPA diet contained 5.5 and 3.5% less CP than the HP diet in the starter and finisher-grower stages, respectively. Belloir et al. (2017) explained that broilers fed an LP (15% CP) diet with high N utilization efficiency showed lower excretion of N.

Tight junctions function as a critical barrier for epithelial defense that protects birds from the translocation of pathogens and allergens and maintains productivity (Ballard et al., 1995). Claudin 1, occludin 1, and ZO 1 are three of the major proteins that regulate the physiological functions of tight junctions in the GIT (Balda et al., 1996; Ulluwishewa et al., 2011). Interestingly, the studies of Ulluwishewa et al. (2011) and Li et al. (2018) further demonstrate that weakening of tight junctions increases intestinal permeability (increased leakage) and leads to elevated serum endotoxin levels. The results of the present study revealed that broilers fed different diets maintained tight junctions in their GITs, based on the nonsignificant changes in serum endotoxin levels and the lack of effect of environmental conditions. Interestingly, LP diet lacks key essential AA which are needed for the production of mucosa as a primary barrier for the pathogenic microorganisms and their products in the GIT. Therefore, we can assume that broilers fed LP diet under poor sanitary conditions compensated the expression of ZO1 owing to an unknown cell signaling pathway to protect barrier function of the GIT in the present study. Similarly, the study by Barekatain et al. (2018) found that broilers fed an LP (17% CP) diet showed higher expression of tight junction genes in the ileum than broilers fed an HP (22% CP) diet, in agreements with the results of the present study. Similarly, Barekatain et al. (2018) mentioned that tight junctions are dynamic structures that can be remodeled by multiple factors such as feed residue, commensal bacteria, and external stimulation. Instead, our results indicated that supplementation of an LP diet with synthetic AA resulted in intermediate expression of tight junction genes (ZO1 and claudin 1) that are required for the maintenance of gut integrity.

In conclusion, LPA diet can be a prospective approach to lower the nitrogen excretion of broiler production while maintaining growth potential of birds. Moreover, observed sustainable growth potentials in LPA diet–fed broilers in poor sanitary conditions during the starter period suggest that LPA diet is the most appropriate one in any environment condition.

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