Revista Brasileira de Zootecnia

Gut microflora and intestinal morphology changes of broiler chickens fed reducing dietary protein supplemented with lysine, methionine, and threonine in tropical environment

Nurhazirah Shazali[1,4] (iD), Teck Chwen Loh [1,4*] (iD), Hooi Ling Foo[2,3] (iD), Anjas Asmara Samsudin[1] (iD)

[1] Universiti Putra Malaysia, Faculty of Agriculture, Department of Animal Science, Serdang, Selangor, Malaysia.
[2] Universiti Putra Malaysia, Faculty of Biotechnology and Biomolecular Sciences, Department of Bioprocess Technology, Serdang, Selangor, Malaysia.
[3] Universiti Putra Malaysia, Institute of Bioscience, Serdang, Selangor, Malaysia.
[4] Universiti Putra Malaysia, Institute of Tropical Agriculture and Food Security, Serdang, Selangor, Malaysia.

*Corresponding author:
tcloh@upm.edu.my

ABSTRACT - This experiment aimed to discover the effect of reducing dietary protein supplemented with lysine, methionine, and threonine on growth performance, volatile fatty acid profile, and intestinal villus height and crypt depth of broilers, as well as the microflora counts isolated from broiler chicken faeces. A total of 288-day-old broilers were allocated to eight treatments with six replicates consisting of 36 birds per treatment. The diets contained dietary protein from 21 to 18% in starter diet and 18 to 16% in finisher diet supplemented with L-lysine, DL-methionine, and L-threonine at the same ratio for all dietary treatments. Body weight and feed intake were determined, and feed conversion ratio was calculated. Blood, intestine, and digesta samples were collected at 21 and 42 days for further analysis. Dietary protein supplemented with amino acids improved growth performance, reduced pathogenic bacteria, and increased beneficial bacteria counts, small intestine villi height and crypt depth, and ileal-digesta volatile fatty acid concentrations of broiler chickens. However, reducing 2% of dietary protein supplemented with lysine, methionine, and threonine showed the best results, especially in growth performance, feed conversion ratio, microflora count, duodenal and jejunal villi height, and ileal-digesta volatile fatty acid concentrations, such as butyric and valeric acids. It is believed that by reducing the level of dietary protein in broiler diet while supplementing with synthetic amino acid may enhance the intestinal morphology, nutrient digestibility, and absorption in broiler chickens and will simultaneously result in better performance.

Keywords: amino acid, broiler, protein supplementation

Introduction

Nowadays, people tend to be concerned with gastrointestinal tract health of broiler chickens due to its improvement for their health and performance (Mountzouris et al., 2007). About 60 to 70% of the overall poultry production cost is related to the price of feedstuffs and, therefore, the preferred poultry feed itself is economically important. The efficient use of feed is extremely important in broiler production (Attia et al., 2012). Dietary protein is a vital growth and reproductive performance regulator for broiler chickens. Nevertheless, it is also essential for the gastrointestinal tract features

and development under a hot climate condition (Laudadio et al., 2012). Furthermore, by reducing the dietary crude protein (CP), it improves dietary CP utilisation and produces heat-tolerant broiler chickens (Tenesa et al., 2016). Laudadio et al. (2012) reported that dietary CP reduction in broiler chicken rations, under hot environmental condition, could be an advantage as compared with the conventional feeding programmes. Previous findings demonstrated that diet with low CP concentration affected growth performance and chemical composition of broiler carcass (Furlan et al., 2004). However, Kamran et al. (2004) reported that reducing the dietary protein up to 20% CP with essential amino acid supplementation led to a better growth performance.

Amino acid requirements vary depending on animal sex, age, and genotype (Ayasan et al., 2009). Moreover, considerable attention was shown in the minimal use of CP in the diet, which helped to influence bacterial ecology of the intestinal tract in different livestock species (Nyachoti et al., 2006). In fact, numerous challenges were encountered when attempting to reduce foodborne pathogens from food of animal origin (Rasekh et al., 2005). Usually, general cross-contamination occurs in a poultry processing plant when the intestinal parts are removed from the birds (Rasekh et al., 2005).

Another issue of concern is the Malaysian climate. Exposure to extreme temperature or environment could be an additional stressor encountered in the tropical environment and contributes to the increasing number of intestinal colonisation and faecal shedding of pathogens in poultry (Bailey, 1988). Additionally, environmental stress may induce the colonisation of pathogenic microbes in animal feed by various factors, such as enteric pathogens, increased pathogen shedding, and carcass contamination during processing (Rigby and Pettit, 1980; Mulder, 1995; Isaacson et al., 1999; Burkholder et al., 2008; Whiley et al., 2013). Furthermore, stress should be an important consideration in a poultry production system, because chickens are routinely subjected to stressors, such as feed withdrawal, temperature fluctuations, and confinement during transportation (Abeyesinghe et al., 2001; St-Pierre et al., 2003; Humphrey, 2006). Overall, animal physiology, health, and productivity may be affected by stress. The gastrointestinal tract is most sensitive to stress, since it may cause a variety of changes, such as alteration of normal, protective microbiota (Tannock and Savage, 1974; Bailey and Coe, 1999), and decreased integrity of the intestinal epithelium (Saunders et al., 1994; Meddings and Swain, 2000; Soderholm et al., 2002).

There is a great interest in the possibility of altering the intestinal microflora and morphology in a favourable way for the host health. Therefore, it is prudent to speculate that reducing dietary protein supply with appropriate amino acid supplementation could reduce the amount of substrate for bacterial proliferation in the gastrointestinal tract, and this would be a great benefit to broiler performance. Therefore, the objective of the current study was to investigate the effect of reducing dietary protein with supplemented lysine, methionine, and threonine on growth performance, faeces microflora count, volatile fatty acid concentration, and gut morphology changes of broiler chickens under tropical climate.

Material and Methods

A total of 288 Cobb 500 broilers, purchased from a local hatchery in Selangor, Malaysia, were raised from 1 to 42 days old. Each cage consisted of six chickens and was randomly allocated to open house cages with in-housed temperature (minimum: 24 °C; maximum: 35 °C) and relative humidity between 65 and 90%. Upon arrival, all chickens were individually tagged with a wing band. At 4 and 21 days old, chickens were vaccinated with Newcastle & Bronchitis vaccine (*MyVAC* 201, Malaysia) through an intraocular route. Furthermore, at seven days old, the chickens were vaccinated with an infectious bursal disease (IBD) vaccine (*MyVAC*, Malaysia). Water and feed were provided *ad libitum.*

The starter (18-21% CP) and finisher (15-18% CP) diets were given to birds from 0 to 21 days old and 22 to 42 days old, supplemented with three essential amino acids (L-lysine, DL-methionine, and L-threonine), respectively. Thus, the eight treatment groups (Tables 1 and 2) were T1: (starter: 21% CP; finisher: 18% CP); T2: (starter: 21% CP; finisher: 18% CP); T3: (starter: 20.5% CP; finisher: 17.5% CP); T4: (starter: 20% CP; finisher: 17% CP); T5: (starter: 19.5% CP; finisher: 16.5 % CP); T6: (starter: 19% CP; finisher: 16% CP); T7: (starter: 18.5% CP; finisher: 15.5% CP); and T8: (starter: 18% CP;

3

Gut microflora and intestinal morphology changes of broiler chickens fed reducing dietary protein...
Shazali et al.

finisher: 15% CP). Diets were formulated based on their nutrient requirements by using least-cost feed formulation software (*FeedLive*, Thailand). All diets were formulated to be isocaloric (3,000 kcal/kg). All diets were supplemented with lysine, methionine, and threonine, except for the control, which was only supplemented with methionine. The control diet received amino acids from corn-soybean diets.

Table 1 - Starter feed formulation and composition of different levels of dietary crude protein diet with lysine, methionine, and threonine supplementation

Ingredient (%)	Dietary treatment[1]							
	T1 (control)	T2	T3	T4	T5	T6	T7	T8
Reduction of crude protein	0.0%	0.0%	0.5%	1.0%	1.5%	2.0%	2.5%	3%
Crude protein (%)	21	21	20.5	20	19.5	19	18.5	18
Corn	49.00	41.40	42.70	41.58	42.02	43.57	44.55	45.19
Crude palm oil	2.95	4.52	4.40	4.60	4.70	4.45	4.40	4.40
Wheat pollard	8.71	14.57	15.00	18.00	19.10	19.25	20.10	21.20
Soy bean meal	29.00	30.60	28.90	26.50	24.75	23.20	21.33	19.47
Fish meal	6.37	4.70	4.70	5.00	5.00	5.00	5.00	5.00
Dicalcium phosphate	0.41	0.61	0.62	0.51	0.50	0.52	0.51	0.51
Calcium carbonate	1.10	1.10	1.10	1.14	1.16	1.16	1.17	1.19
Vitamin premix[2]	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Mineral premix[3]	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Salt	0.23	0.23	0.23	0.23	0.23	0.23	0.23	0.23
Antioxidant	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
Toxin binder	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14
L-lysine	0.00	0.00	0.05	0.10	0.15	0.20	0.26	0.31
DL-methionine	0.09	0.10	0.11	0.12	0.12	0.13	0.14	0.15
L-threonine	0.00	0.02	0.05	0.09	0.12	0.14	0.17	0.20
Total	100	100	100	100	100	100	100	100
Calculated and determined composition[4] (%)								
Crude protein	21.1	21.0	20.5	20.0	19.5	19.0	18.5	18.0
Energy (kcal/kg)	3004	3006	3007	3001	3007	3003	3004	3005
Fat	5.46	6.60	6.51	6.67	6.77	6.56	6.53	6.53
Fibre	3.99	4.35	4.29	4.33	4.30	4.25	4.21	4.18
Calcium	1.05	1.01	1.01	1.00	1.00	1.00	1.00	1.00
Available phosphorus	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Arginine	1.38	1.34	1.29	1.22	1.16	1.12	1.06	1.00
Lysine	1.21	1.21	1.20	1.20	1.20	1.20	1.20	1.20
Methionine + cysteine	0.78	0.79	0.78	0.78	0.77	0.76	0.75	0.74
Methionine	0.46	0.46	0.46	0.46	0.46	0.46	0.46	0.46
Threonine	0.80	0.79	0.79	0.79	0.79	0.79	0.79	0.79
Tryptophan	0.26	0.25	0.24	0.20	0.20	0.21	0.20	0.19
Lysine:methionine	2.61	2.62	2.61	2.61	2.61	2.61	2.60	2.60
Lysine:threonine	1.50	1.53	1.52	1.52	1.52	1.52	1.52	1.52

[1] T1: 21% CP without lysine and threonine supplementation (control); T2: 21% CP with lysine, methionine, and threonine supplementation; T3: 20.5% CP with lysine, methionine, and threonine supplementation; T4: 20% CP with lysine, methionine, and threonine supplementation; T5: 19.5% CP with lysine, methionine, and threonine supplementation; T6: 19% CP with lysine, methionine, and threonine supplementation; T7: 18.5% CP with lysine, methionine, and threonine supplementation; T8: 18% CP with lysine, methionine, and threonine supplementation.
[2] Provided per kg of diet: Fe, 120 mg; Mn, 150 mg; Cu, 15 mg; Zn, 120 mg; I, 1.5 mg; Se, 0.3 mg; Co, 0.4 mg.
[3] Provided per kg diet: vitamin A, 11,494 IU; vitamin D, 1,725 IU; vitamin E, 40 IU; vitamin K3, 2.29 mg; cobalamin, 0.05 mg; thiamine, 1.43 mg; riboflavin, 3.44 mg; folic acid, 0.56 mg; biotin, 0.05 mg; pantothenic acid, 6.46 mg; niacin, 40.17 mg; pyridoxine, 2.29 mg.
[4] The diets were formulated using Feedlive International Software (Thailand).

4

Gut microflora and intestinal morphology changes of broiler chickens fed reducing dietary protein...
Shazali et al.

Methionine levels in corn-soybean diets were inadequate to support broiler requirements. The amino acids for starter and finisher diets in different treatments were adjusted to a similar ratio of lysine and methionine to maintain the level at or above, according to the Cobb Broiler Management Guide

Table 2 - Finisher feed formulation and composition of different levels of dietary crude protein (CP) with lysine, methionine, and threonine supplementation

Ingredient (%)	Dietary treatment[1]							
	T1 (control)	T2	T3	T4	T5	T6	T7	T8
Reduction of crude protein	0.0%	0.0%	0.5%	1.0%	1.5%	2.0%	2.5%	3%
Crude protein (%)	18	18	17.5	17	16.5	16	15.5	15
Corn	55.00	49.50	49.25	51.00	51.51	52.25	52.86	54.50
Crude palm oil	3.75	4.95	5.20	4.95	5.00	5.00	5.00	4.80
Wheat pollard	7.23	14.91	16.00	15.76	16.70	17.20	18.30	18.48
Soybean meal	25.00	22.00	21.70	20.34	19.21	18.00	16.40	14.50
Fish meal	5.00	4.50	3.40	3.40	2.80	2.50	2.30	2.50
Dicalcium phosphate	0.37	0.33	0.54	0.56	0.67	0.83	0.83	0.83
Calcium carbonate	1.10	1.15	1.16	1.16	1.18	1.18	1.18	1.18
Vitamin premix[2]	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Mineral premix[3]	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Salt	0.30	0.23	0.23	0.23	0.24	0.25	0.25	0.25
Antioxidant	0.02	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Toxin binder	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14
L-lysine	0.00	0.09	0.14	0.19	0.24	0.29	0.35	0.40
DL-methionine	0.09	0.11	0.13	0.13	0.15	0.16	0.17	0.17
L-threonine	0.00	0.08	0.12	0.13	0.17	0.19	0.22	0.25
Total	100	100	100	100	100	100	100	100
Calculated and determined composition[4] (%)								
Crude protein	18.0	18.0	17.5	17.0	16.5	16.0	15.5	15.0
Energy (kcal/kg)	3108	3102	3103	3102	3101	3100	3100	3101
Fat	6.14	7.21	7.36	7.16	7.17	7.16	7.15	7.01
Fibre	3.66	3.97	4.02	3.95	3.95	3.92	3.90	3.83
Calcium	0.90	0.90	0.90	0.91	0.91	0.94	0.92	0.93
Available phosphorus	0.45	0.45	0.45	0.45	0.45	0.46	0.46	0.46
Arginine	1.21	1.08	1.03	0.99	0.94	0.89	0.83	0.78
Lysine	1.03	1.05	1.05	1.05	1.05	1.05	1.05	1.05
Methionine + cysteine	0.63	0.72	0.71	0.70	0.70	0.69	0.68	0.67
Methionine	0.34	0.43	0.43	0.43	0.43	0.43	0.43	0.43
Threonine	0.72	0.72	0.72	0.72	0.72	0.72	0.72	0.72
Tryptophan	0.22	0.20	0.19	0.19	0.17	0.17	0.16	0.15
Lysine:methionine	3.03	2.44	2.44	2.45	2.44	2.44	2.44	2.44
Lysine:threonine	1.44	1.46	1.46	1.46	1.46	1.46	1.46	1.46

[1] T1: starter: 21% CP; finisher: 18% CP without lysine and threonine supplementation (control).
T2: starter: 21% CP; finisher: 18% CP with lysine, methionine, and threonine supplementation.
T3: starter: 20.5% CP; finisher: 17.5% CP with lysine, methionine, and threonine supplementation.
T4: starter: 20% CP; finisher: 17% CP with lysine, methionine, and threonine supplementation.
T5: starter: 19.5% CP; finisher: 16.5% CP with lysine, methionine, and threonine supplementation.
T6: starter: 19% CP; finisher: 16% CP with lysine, methionine, and threonine supplementation.
T7: starter: 18.5% CP; finisher: 15.5% CP with lysine, methionine, and threonine supplementation.
T8: starter: 18% CP; finisher: 15% CP with lysine, methionine, and threonine supplementation.
[2] Provided per kg of diet: Fe, 120 mg; Mn, 150 mg; Cu, 15 mg; Zn, 120 mg; I, 1.5 mg; Se, 0.3 mg; Co, 0.4 mg.
[3] Provided per kg diet: vitamin A, 11,494 IU; vitamin D, 1,725 IU; vitamin E, 40 IU; vitamin K3, 2.29 mg; cobalamin, 0.05 mg; thiamine, 1.43 mg; riboflavin, 3.44 mg; folic acid, 0.56 mg; biotin, 0.05 mg; pantothenic acid, 6.46 mg; niacin, 40.17 mg; pyridoxine, 2.29 mg.
[4] The diets were formulated using Feedlive International Software (Thailand).

5

Gut microflora and intestinal morphology changes of broiler chickens fed reducing dietary protein...
Shazali et al.

(Cobb-Vantress, 2013). The L-lysine HCl, DL-methionine, and L-threonine used in the diets were of feed grade and were formulated based on total amino acids required for the chickens. The concentrations of dietary calcium and available phosphorus were equally maintained in all treatments. This experiment was conducted for six weeks, and feed intake was measured weekly by deducting the feed balance from the original quantity supplied to the chickens.

Faecal lactic acid bacteria (LAB) and *Enterobacteriaceae* (ENT) population were determined by using a method described by Foo et al. (2003), Loh et al. (2009), and Shazali et al. (2014). The faecal sample was homogeneously mixed at a ratio of 1 g sample to 9 mL of peptone water in a universal bottle and incubated for 1 h at room temperature. The samples were then subjected to a series of 10-fold dilution by using 0.1% (v/v) peptone water, and 0.1 mL of each appropriately diluted sample was then plated onto *Lactobacillus* agar DE Man, ROGOSA, and SHARPE (MRS-agar, Merck, KgaA, Darmstadt, Germany) and incubated under anaerobic condition at 30 °C for 48 h. However, ENT was spread plated and enumerated on Eosin-methylene-blue lactose sucrose agar plates (EMB-agar, Merck, KgaA, Darmstadt, Germany) and aerobically incubated for 24 h at 37 °C. The colony-forming unit (cfu) per gram of sample was expressed as a logarithm at base of 10 (Log 10 cfu/g). The enumeration of LAB/ENT was conducted in triplicates. Intestinal morphology changes procedure was applied as described by Choe et al. (2012) and Tenesa et al. (2016). Segments of 5 cm long were removed from the small intestines. The intestinal segments were flushed with 10% neutral buffered formalin solution and then used for the morphometric analysis. Then, the segments were fixed in 10% neutral buffered formalin solution and kept overnight. Intestinal samples were then excised and dehydrated with a series of alcohol in the tissue-processing machine (Leica Microsystems K. K., Tokyo, Japan) and embedded in paraffin wax. Sections of 4 μm were then stained with haematoxylin and eosin, and later examined under light microscope. The distance from the villus tip to villus-crypt junction represented the villus height, while crypt depth was defined as the depth of invagination between adjacent villi. The villi height and crypt depth were measured by using an image analyser.

Volatile fatty acid (VFA) concentrations in the ileal-digesta sample were determined according to the method outlined in Loh et al. (2014). One gram of ileal-digesta sample was mixed with 1 mL of 24% methaphosphoric acid, diluted in 1.5 M sulphuric acid, and kept overnight at room temperature. The internal standard 20 mM 4-methyl-valeric acid was added to the supernatant to achieve 10 mM in combination and stored at −20 °C until gas liquid chromatography (GLC) analysis. Volatile fatty acid was separated by using a silica capillary column in gas-liquid chromatography occupied with a flame ionisation detector.

Blood from two broilers in each cage was collected in ethylenediaminetetraacetic acid (EDTA) tubes. Plasma was separated following a centrifuge at 3000 rpm for 15 min and stored at −20 °C until further analysis. The plasma IgG and IgM concentrations were determined by using chicken IgM and IgG ELISA Kits (Alpha Diagnostic International). Four hundred and fifty nanometre wavelength was used to measure wavelength by using a microplate reader (Model 3550-UV, Bio-Rad). The concentrations of plasma IgM and IgG were calculated by using standard curves.

All data were analysed based on a completely randomised design by using the General Linear Model procedure of the SAS (Statistical Analysis System, version 9.2) software. Duncan's multiple range tests were used to compare mean at P<0.05 of treatments. Data were presented as the mean ± standard error of the mean (SEM).

Results

All diets supplemented with essential amino acids had significantly quadratically increased (quadratic, P<0.05) body weight (BW), average daily gain (ADG), and total weight gain (TWG) as compared with the control diet (T1) (Table 3). As for the FCR, control diet presented the highest value among all treatments. However, reduction of CP up to 2% showed a lower (P<0.05) FCR among all dietary treatments. The overall results showed by reducing CP level up to 2% had a better growth performance as compared with other treatments.

6

Gut microflora and intestinal morphology changes of broiler chickens fed reducing dietary protein...
Shazali et al.

Faecal LAB and ENT population were not affected (P>0.05) by reduction of CP level in the broiler diet (Table 4). The highest (P<0.05) faecal LAB population was observed in chickens fed 0% reduction of CP with equal amino acids supplementation (T2) as compared with other treatments. As for faecal ENT populations, by reducing 2% of CP in the diets and supplemented with amino acids (T6) had lowest (P<0.05) faecal ENT populations than the other treatments. As for the LAB to ENT ratio, reduction of 2% CP shows the highest (P<0.05) ratio compared with other treatments.

The most important VFA for broiler chickens are acetic, propionic, and butyric acids. Total VFA was significantly quadratically increased (quadratic; P<0.05) with decreasing levels of CP as compared with

Table 3 - Growth performance of birds fed different levels of dietary crude protein (CP) supplemented with lysine, methionine, and threonine at 6 weeks of age

| Parameter | Dietary treatment[1] | | | | | | | | SEM | Contrast; P-values | |
	T1 (control)	T2	T3	T4	T5	T6	T7	T8		Linear	Quadratic
Reduction of crude protein	0.0%	0.0%	0.5%	1.0%	1.5%	2.0%	2.5%	3%			
Body weight (kg)	1.74c	2.05b	2.05b	2.01b	2.01b	2.15a	2.03b	2.02b	0.26	<.0001	<.0001
Average daily gain (g)	40.4c	47.4b	47.95ab	46.51b	46.49b	49.81a	47.07b	46.64b	0.67	<.0001	<.0001
Feed intake (kg)	3.15a	3.32a	3.33a	3.32a	3.29a	3.41a	3.25a	3.27a	0.08	0.41	0.30
Total weight gain (kg)	1.68c	1.99b	2.02ab	1.95b	1.95b	2.09a	1.98b	1.96b	0.67	<.0001	<.0001
Feed conversion ratio	1.87a	1.67ab	1.65ab	1.7ab	1.68ab	1.63b	1.66ab	1.67ab	0.19	0.13	0.09

SEM - standard error of the mean.
[1] T1: starter: 21% CP; finisher: 18% CP without lysine and threonine supplementation (control).
 T2: starter: 21% CP; finisher: 18% CP with lysine, methionine, and threonine supplementation.
 T3: starter: 20.5% CP; finisher: 17.5% CP with lysine, methionine, and threonine supplementation.
 T4: starter: 20% CP; finisher: 17% CP with lysine, methionine, and threonine supplementation.
 T5: starter: 19.5% CP; finisher: 16.5% CP with lysine, methionine, and threonine supplementation.
 T6: starter: 19% CP; finisher: 16% CP with lysine, methionine, and threonine supplementation.
 T7: starter: 18.5% CP; finisher: 15.5% CP with lysine, methionine, and threonine supplementation.
 T8: starter: 18% CP; finisher: 15% CP with lysine, methionine, and threonine supplementation.
abc - Means in the row with different letters are significantly different (P<0.05).

Table 4 - Faecal lactic acid bacteria and *Enterobacteriaceae* counts of birds fed different levels of dietary crude protein (CP) supplemented with lysine, methionine, and threonine

| Parameter | Dietary Treatment[1] | | | | | | | | SEM | Contrast; P-values | |
	T1 (control)	T2	T3	T4	T5	T6	T7	T8		Linear	Quadratic
Reduction of crude protein	0.0%	0.0%	0.5%	1.0%	1.5%	2.0%	2.5%	3%			
Lactic acid bacteria (LAB; Log_{10} cfu/g)	6.77cd	7.97a	7.35b	7.48b	6.65cd	7.33b	6.42d	7.14bc	0.34	0.49	0.81
Enterobacteriaceae (ENT; Log_{10} cfu/g)	6.62ab	6.56ab	6.35bc	6.05c	6.18c	5.48d	6.78a	6.71ab	0.01	0.22	0.77
LAB:ENT ratio	1.02de	1.22b	1.16bc	1.23b	1.08cd	1.34a	0.94e	1.06cd	0.38	0.07	0.66

SEM - standard error of the mean.
[1] T1: starter: 21% CP; finisher: 18% CP without lysine and threonine supplementation (control).
 T2: starter: 21% CP; finisher: 18% CP with lysine, methionine, and threonine supplementation.
 T3: starter: 20.5% CP; finisher: 17.5% CP with lysine, methionine, and threonine supplementation.
 T4: starter: 20% CP; finisher: 17% CP with lysine, methionine, and threonine supplementation.
 T5: starter: 19.5% CP; finisher: 16.5% CP with lysine, methionine, and threonine supplementation.
 T6: starter: 19% CP; finisher: 16% CP with lysine, methionine, and threonine supplementation.
 T7: starter: 18.5% CP; finisher: 15.5% CP with lysine, methionine, and threonine supplementation.
 T8: starter: 18% CP; finisher: 15% CP with lysine, methionine, and threonine supplementation.
abcde - Means ± SEM in the row with different letters are significantly different at P<0.05.

7

Gut microflora and intestinal morphology changes of broiler chickens fed reducing dietary protein...
Shazali et al.

the negative control (Table 5). However, individual VFA was not affected (P>0.05) by the different levels of CP in the diets. Reduction of 0.5% CP (T3) led to higher (P<0.05) acetic acid concentration compared with other dietary treatments. Moreover, the diet with decreased 2.5% CP with equal amino acid supplementation (T7) had a significantly higher (P<0.05) propionic concentration as compared with other treatments. Furthermore, the highest (P<0.05) butyric acid concentration was observed in diet containing less than 2% CP as compared with other treatments. In terms of total VFA concentration, the reduction of 2.5% CP in the diet showed the highest (P<0.05) total VFA concentration as compared with all treatments.

The duodenal, jejunal, and ileal of chickens fed lower CP levels quadratically increased (quadratic; P<0.05) villi height with reduced CP levels in the diets (Table 6); meanwhile, the highest (P<0.05) duodenal and jejunal villi heights were observed in chickens fed diet containing less than 2% CP with equal amino acid supplementation as compared with the other treatments. In the case of ileal villi height, 0% reduction CP level with equal amino acid supplementation was significantly higher (P<0.05) as compared with other treatments. Concerning crypt depth, the duodenal crypt was linearly shallow (P<0.05) with reduction of CP in the diets. Howbeit, jejunal and ileal crypt depth was not affected by the different levels of CP. In terms of jejunal crypt depth, chickens fed the control diet had significantly deeper (P<0.05) jejunal crypt depth than chickens fed other diets. There was significant difference (P<0.05) in 0% reduction CP level with equal amino acid supplementation in the ileal crypt depth as compared with all treatment chickens. Furthermore, a significant quadratic increase (quadratic; P<0.05) was observed in the duodenal and jejunal villi height to crypt depth ratio with the reduction of CP in the diets. In contrast, ileal villi height to crypt depth ratio was linearly increased with the different levels of CP. The lowest villus height to crypt depth ratio was observed in chickens fed the control as compared with other treatments.

Plasma IgG showed a significant quadratic increase (quadratic; P<0.05) with the reduction of CP levels (Figure 1). Blood plasma of chickens fed the T6 showed significantly higher (P<0.05) IgG concentration than other treatments. On the other hand, plasma IgM was not affected by the reduction of CP in the diets. Reduction of 2% CP in the dietary treatment had significantly higher (P<0.05) plasma IgM than other dietary treatments.

Table 5 - Concentration of volatile fatty acids (VFA) of birds fed different levels of dietary crude protein (CP) supplemented with lysine, methionine, and threonine

Volatile fatty acid (mM/L)	Dietary treatment[1]								SEM	Contrast; P-values	
	T1 (control)	T2	T3	T4	T5	T6	T7	T8		Linear	Quadratic
Reduction of crude protein	0.0%	0.0%	0.5%	1.0%	1.5%	2.0%	2.5%	3%			
Acetic	17.6bc	17.7bc	41.9a	20.9bc	23.4bc	21.2bc	11.8c	33.9ab	0.18	0.40	0.06
Propionic	20.9bc	29.0b	17.9c	25.4bc	30.4b	24.0bc	40.8a	18.6c	0.05	0.11	0.29
Iso-butyric	14.3a	14.1a	15.6a	16.9a	14.5a	13.2a	13.8a	13.4a	0.01	0.48	0.43
Butyric	11.9abc	13.7abc	10.9c	11.7bc	13.2abc	17.0a	16.3ab	3.8d	0.02	0.91	0.64
Iso-valeric	6.1b	7.2ab	7.5ab	5.4b	5.7b	8.2ab	9.7a	5.4b	0.00	0.32	0.26
Valeric	5.4bc	6.5abc	5.3bc	7.9a	7.1ab	8.1a	4.5c	7.3ab	0.01	0.07	0.95
Total VFA	64.5c	87.1ab	91.6ab	86.4ab	92.5ab	81.9b	96.9a	80.6b	0.11	0.00	0.01

SEM - standard error of the mean.
[1] T1: starter: 21% CP; finisher: 18% CP without lysine and threonine supplementation (control).
   T2: starter: 21% CP; finisher: 18% CP with lysine, methionine, and threonine supplementation.
   T3: starter: 20.5% CP; finisher: 17.5% CP with lysine, methionine, and threonine supplementation.
   T4: starter: 20% CP; finisher: 17% CP with lysine, methionine, and threonine supplementation.
   T5: starter: 19.5% CP; finisher: 16.5% CP with lysine, methionine, and threonine supplementation.
   T6: starter: 19% CP; finisher: 16% CP with lysine, methionine, and threonine supplementation.
   T7: starter: 18.5% CP; finisher: 15.5% CP with lysine, methionine, and threonine supplementation.
   T8: starter: 18% CP; finisher: 15% CP with lysine, methionine, and threonine supplementation.
abcde - Means ± SEM in the row with different letters are significantly different at P<0.05.

8

Gut microflora and intestinal morphology changes of broiler chickens fed reducing dietary protein...
Shazali et al.

Table 6 - Villus Height and crypt depth in small intestine of birds fed different levels of dietary crude protein (CP) supplemented with lysine, methionine, and threonine

| Parameter | Dietary treatment[1] | | | | | | | | | Contrast; P-values | |
	T1 (control)	T2	T3	T4	T5	T6	T7	T8	SEM	Linear	Quadratic
Reduction of crude protein	0.0%	0.0%	0.5%	1.0%	1.5%	2.0%	2.5%	3%			
Villus height (μm)											
Duodenum	898.08d	1776.66a	1666.34ab	1434.43c	1533.74bc	1814.35a	1622.86abc	1674.02ab	0.75	0.002	<0.001
Jejunum	671.54d	963.52a	849.51bc	881.75b	863.63bc	983.27a	731.95d	810.96c	0.92	0.001	<0.001
Ileum	474.41c	718.61a	629.18b	651.49b	656.53b	633.27b	664.14b	651.51b	0.61	0.0002	<0.001
Crypt depth (μm)											
Duodenum	166.89ab	168.89a	147.02dc	155.68abcd	142.74d	152.46bcd	162.80abc	164.70ab	0.42	0.003	0.971
Jejunum	176.93a	172.15bc	149.75d	177.28a	159.99bcd	171.89abc	172.89ab	158.62cd	0.36	0.052	0.242
Ileum	85.03c	93.39a	81.79d	86.34bc	88.11bc	87.09bc	88.34b	86.34bc	0.15	0.752	0.384
Villus height:crypt depth ratio (μm)											
Duodenum	4.81d	11.00b	11.69a	11.17ab	10.51b	10.53b	10.52b	9.49c	0.14	<0.001	<0.001
Jejunum	3.57c	5.67a	5.51a	4.87b	5.34ab	5.51a	5.25ab	4.80b	0.08	0.0002	<0.001
Ileum	5.74b	7.59a	7.51a	7.61a	7.81a	7.27a	7.46a	7.31a	0.07	<0.001	0.0002

SEM - standard error of the mean.
[1] T1: starter: 21% CP; finisher: 18% CP without lysine and threonine supplementation (control).
  T2: starter: 21% CP; finisher: 18% CP with lysine, methionine, and threonine supplementation.
  T3: starter: 20.5% CP; finisher: 17.5% CP with lysine, methionine, and threonine supplementation.
  T4: starter: 20% CP; finisher: 17% CP with lysine, methionine, and threonine supplementation.
  T5: starter: 19.5% CP; finisher: 16.5% CP with lysine, methionine, and threonine supplementation.
  T6: starter: 19% CP; finisher: 16% CP with lysine, methionine, and threonine supplementation.
  T7: starter: 18.5% CP; finisher: 15.5% CP with lysine, methionine, and threonine supplementation.
  T8: starter: 18% CP; finisher: 15% CP with lysine, methionine, and threonine supplementation.
abcd - Means ± SEM in the row with different letters are significantly different (P<0.05).
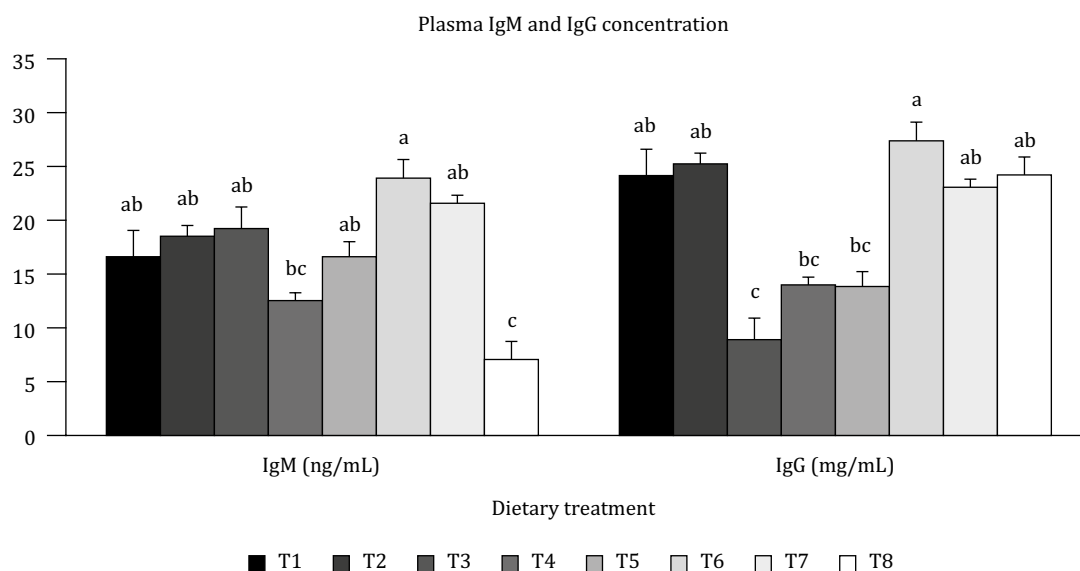
a-c - Different alphabets between means indicate significant difference at P<0.05.
T1: starter: 21% CP; finisher: 18% CP without lysine and threonine supplementation (control).
T2: starter: 21% CP; finisher: 18% CP with lysine, methionine, and threonine supplementation.
T3: starter: 20.5% CP; finisher: 17.5% CP with lysine, methionine, and threonine supplementation.
T4: starter: 20% CP; finisher: 17% CP with lysine, methionine, and threonine supplementation.
T5: starter: 19.5% CP; finisher: 16.5% CP with lysine, methionine, and threonine supplementation.
T6: starter: 19% CP; finisher: 16% CP with lysine, methionine, and threonine supplementation.
T7: starter: 18.5% CP; finisher: 15.5% CP with lysine, methionine, and threonine supplementation.
T8: starter: 18% CP; finisher: 15% CP with lysine, methionine, and threonine supplementation.

Figure 1 - Plasma immunoglobulin concentration of broilers fed different levels of crude protein (CP) supplemented with essential amino acids.

9

Gut microflora and intestinal morphology changes of broiler chickens fed reducing dietary protein...
Shazali et al.

Discussion

In this experiment, reduction of CP up to 2% from the normal broiler CP requirement and supplementation with available amino acid improved body weight, average daily gain, feed intake, and feed conversion ratio in broiler chickens (Table 3). However, there were no significant differences in growth performance between the 0% CP level reduction with equal amino acid supplementation and the 2% reduced CP in the dietary treatment. These results agree with Han and Lee (2000), who reported that a reduction of 2% CP supplemented with 0.1% extra lysine did not affect broiler chicken performance. Better performance of animals fed the low dietary CP supplemented with amino acid could be due to the fulfilment of basic amino acid and CP requirement in the dietary treatments (Banerjee et al., 2013). In previous studies, many researchers reported a reduced CP level with crystalline amino acid supplementation in broiler diets without affecting the chicken performance. Lysine is the first limiting amino acids in common broiler diets, and its requirements for broilers are higher in low-protein diets for maximum weight gain and feed efficiency (Labadan et al., 2001; Corzo et al., 2002; Saima et al., 2010). Threonine is considered as the third limiting essential amino acid in low-CP diets (Kidd et al., 1999; Baylan et al., 2006; Ayasan et al., 2009). To balance the broiler amino acid requirement, L-threonine was added to the low-protein diets (Ayasan et al., 2009). The studies included 22.7% CP (Waldroup et al., 2005), 17.6% CP (Corzo et al., 2005), 19% CP (Namroud et al., 2008), and 16% CP (Aletor et al., 2000), respectively, in the starter diets and 18.2% CP (Dari et al., 2005), and 16% CP (Gomide et al., 2012) in finisher diets. In this study, the growth performance of broilers in T6 (19% CP in starter and 16% CP in finisher diet) supplemented with essential amino acid had higher body weight gain, average daily gain, and feed intake and lower feed conversion ratio as compared with the 21% CP in starter and 18% CP in finisher diet supplemented with amino acids. Furthermore, Saima et al. (2010) reported that CP levels did not significantly affect the weight gain of broilers until four weeks of age in hot climate condition.

Nevertheless, the levels of lysine formulated with corn and soybean meal diet had a significant effect on the live weight gain and the feed conversion ratio of broiler (Oliveira et al., 2013). Dietary methionine and lysine deficiency were shown to impair chicken growth (Zhai et al., 2016a). According to Zhai et al. (2016b), the highest supplemental levels of methionine and lysine in broiler diets were not affected by the feed intake, but body weight was increased, and this resulted in a lower feed conversion ratio of broiler chickens. Ayasan and Okan (2010) also mentioned that threonine and lysine supplementation to broiler diets improved feed intake and body weight gain. Nevertheless, supplemented threonine and lysine did not affect their feed conversion ratio (Rezaeipour and Gazani, 2014). This outcome was different from that reported by Bregendahl et al. (2002), who found that decreasing the dietary CP resulted in a decreased average daily gain, feed efficiency, and growth rate of broiler chickens due to slow growth, lower feed efficiency, and less nitrogen retention than chickens fed the commercial diet (Bregendahl et al., 2002). In commercial feeds, diets are always formulated to minimum CP levels, and consequently, amino acids will be supplemented at a higher level than required (de Beer, 2011). However, increase in threonine to lysine ratio in the diets improved broiler FCR and increased feed intake and weight gain compared with the lower level of threonine, and that showed a significant difference in weight gain of *Clostridium*-infected chickens (Valizade et al., 2016).

Previously, Bowmaker and Gous (1991) mentioned that amino acid composition in the diet was crucially important for broiler chicken performance, suggesting that CP requirement could be reduced once the amino acid requirements were met (Lopez and Leeson, 1994). Moran Jr. (2007) mentioned that deficiency of amino acids may cause marginal deficiencies in the offspring. On the other hand, Wilson (1997) stated that higher levels of a particular nutrient in the diets, particularly amino acids, could be deposited and may cause health problem due to toxicity. Defective body weights and greater FCR were found in the chickens offered the dietary CP lower than 22%. In this study, 2% lower dietary CP supplemented with synthetic amino acids had similar growth compared with the 0% reduction CP level with equal amino acid supplementation. This result indicated that amino acid supplementation could be used to replace and meet the amino acid loss that originated from dietary CP (Jiang et al., 2005).

10

Gut microflora and intestinal morphology changes of broiler chickens fed reducing dietary protein...
Shazali et al.

Previous studies conducted by Waldroup et al. (2005) claimed that the supplementation of synthetic amino acids could partially improve the loss in body weight gain.

In this study, faecal LAB count in chickens fed 0% reduction CP level with equal amino acid supplementation diet was significantly different as compared with the other treatments (Table 4). Control (T1) showed a higher ENT count as compared with other treatments. However, reduction of 2% CP in the diets had a slightly lower ENT count than other amino acid supplementation in the low CP diets. This result was in agreement with Laudadio et al. (2012), who claimed that reduction of CP up to 18.5% decreased faecal pH values and decreased the number of pathogenic bacteria, such as aerobic mesophilic and *Escherichia coli* in broiler excreta. However, Nyachoti et al. (2006) claimed that the reduction of 23 to 17% CP did not affect the microbial counts in both beneficial and pathogenic bacteria in ileal digesta. Additionally, diet with low CP did not influence the coliforms and *Lactobacilli* counts in jejunum and colon of the chickens (Bikker et al., 2006). Generally, the increase in *Lactobacilli* counts may help suppress the coliform counts, and this could be attributed to the microbial colonisation resistance in the small intestine (Bikker et al., 2006). Thus, the increased *Lactobacilli* population may reduce the challenge of the *Lactobacilli* population to fight for space and nutrient availability in the intestinal tracts (Bikker et al., 2006). This finding is similar to our current results by showing that lactic acid-producing bacteria suppress pathogenic bacteria activities, such as *Enterobacteriaceae*. The result showed that reducing the dietary CP up to 2% and supplementing with amino acids increased LAB and reduced ENT population. This may improve and provide a favourable environment in the intestinal tract. The favourable environment will contribute to higher nutrient digestibility and absorption that enhance the chicken to grow subsequently, recording a better growth performance. Thus, it also contributes to reduce foodborne pathogens from animal origin foods (Rasekh et al., 2005).

Volatile fatty acids (VFA) consist of carbon chain molecules and some called short chain fatty acids. Common compounds in VFA are acetic, propionic, and butyrate acids. These compounds are typically absorbed by the inner lining of the intestine and pass into the bloodstream before passing through the liver. Acetic acid is often used to build energy, as well as create lipids. The liver will not use much of the acetic acid, but propionic acid is generally removed from the blood by the organ. Bikker et al. (2006) reported that butyric acid acts as a microbial energy source and maintains the structure and function of human epithelial cells. Generally, VFA concentrations indicate the microbial activity along the small intestine and, usually, VFA concentration is higher in ileal digesta than in the jejunum and duodenum.

Franklin et al. (2002) observed similar findings and reported that the increased VFA concentration in the cecum coincided with the increased number of microbial populations in the cecum. Bacteria often produce VFA in the intestines by digesting cellulose. Cherian et al. (2013) reported that the growth of the pathogen in the gut could be inhibited by lowering the gut pH (Loh et al., 2003). Pluske et al. (2003) claimed that by lowering the dietary CP content, a reduction in fermentation and formation of the microbial metabolite in the ileum and cecum could be expected. The results of this study showed that by lowering the dietary CP there is a reduction in nitrogen and fermentable energy supply to the intestinal microbes. Another study showed that lowering the dietary CP reduces the ammonia concentrations (Bikker et al., 2006; Nyachoti et al., 2006) and individual VFA (Nyachoti et al., 2006) in ileal digesta of pigs weaned at 18 days old. In this study, the dietary CP content did not affect the total concentrations of VFA in ileal digesta but showed a higher (P<0.05) concentration in the dietary CP supplemented with amino acids as compared with the dietary CP without supplementation of amino acids (Table 5). Reducing dietary CP with amino acid supplementation resulted in significant linear reductions in VFA in ileal digesta (Franklin et al., 2002). However, in this study, reducing CP in the diet and supplementing it with amino acids contributed to fluctuated results in the total VFA concentration. In addition, VFA concentration was not consistent among the dietary CP studies. Htoo et al. (2007) speculated that the absence of an effect of dietary CP content on the concentration of VFA in the ileum might be affected by the rapid passage rate of digesta through the small intestine, lack of bacterial fermentation occurring in the ileum part, and nutritive value and nutrient digestibility of certain selected feed ingredients. Furthermore, Bikker et al. (2006) reported that by reducing the 21.1% dietary CP to 15.3% does not influence the individual and total VFA in the ileal digesta of pigs weaned at 26 days old.

11

Gut microflora and intestinal morphology changes of broiler chickens fed reducing dietary protein...
Shazali et al.

An indirect relation between lactic acid bacteria and butyrate was suggested by Tsukahara et al. (2002), whereas the higher counts of lactic acid bacteria produced more lactic acid and were converted into butyrate in the lumen. Hence, the current result showed that by reducing the dietary CP supplemented with amino acids had no significant difference in total VFA concentrations as compared with dietary CP without amino acid supplementation. This may create a favourable environment for beneficial bacteria and better nutrient digestibility of broiler chickens.

Small intestine consists of the duodenum, jejunum, and ileum, which they help to absorb nutrients from feed. The longer the length of villi, the higher is the value of nutrient absorption. Lower villus height results in decreased intestinal absorption area. Gut morphology plays an important role in dietary nutrient absorption efficiency and can be determined by measuring the villi height and crypt depth of the intestine (Swatson et al., 2002). The height of villi and depth of crypt are mostly depended on the diet provided (Incharoen and Yamauchi, 2009; Incharoen et al., 2009). In the present study, reducing dietary CP up to 2% plus supplementation with amino acids led to better duodenum and jejunum villi height as compared with the other treatments (Table 6). Thus, this result was in agreement with Sritiawthai et al. (2013), who claimed that ducks that received 18% CP also had higher villus height in duodenum and jejunum as compared with higher CP levels. However, this result contradicted Abbasi et al. (2014), who found that a reduction in dietary CP led to significantly lowered jejunal villi height and shallowed crypt depth in broiler chickens. Furthermore, Abbasi et al. (2014) reported that decrease in CP diet had a similar effect on the villi height to crypt depth ratio, whereby this result also contradicted our findings. Gu and Li (2004) reported that increasing dietary CP caused a higher number of goblet cell in the distal jejunum of a piglet that was associated with the development of villus height, crypt depth, and epithelium cell size. It is believed that high dietary CP rewards the small intestinal mucosa with higher digestive and absorptive capacity compared with low dietary CP in piglet (Gu and Li, 2004). Wu (1998) also pointed out that small intestine may use about 30 to 50% of essential amino acids. In addition, some of the amino acids were not available for extra intestinal tissue (Wu, 1998). Schaart et al. (2005) found that dietary threonine was utilised by small intestine mucosa for protein synthesis, which was related to a decrease of threonine levels in faecal samples.

Among the essential amino acids, threonine is important for maintaining the gut barrier integrity and has an important role in the structure and function of gastrointestinal tract (Valizade et al., 2016). Threonine participates in protein synthesis, generates metabolic products such as glycine and serine, and serves as a component of body protein for broiler chickens (Ayasan et al., 2009). Production of glycine and serine derived from threonine catabolism is needed in gastrointestinal mucin production (Lemme, 2003). Law et al. (2000) and Ball (2001) reported that piglets that received deficient diets in threonine had lower intestinal weight and developed less intestinal structure. In the current study, the results are in agreement with Law et al. (2000) and Ball (2001), whereby different intestinal structures were found in different dietary CP supplemented with essential amino acids. Wu et al. (1996) mentioned that an increase in glutamic supplementation prevented jejunal atrophy as indicated by villus height. At the same time, it also increased the gain:feed ratio. However, in our study, amino acid supplementation in the low dietary CP showed significantly different villi height and crypt depth of duodenum, jejunum, and ileum as compared with dietary CP without amino acid supplementation. However, by reducing dietary CP up to 2% and supplementing with amino acids led to higher duodenal and jejunal villi height and crypt depth as compared with other dietary CP supplemented with amino acids.

The interaction between nutrition and immunity showed an implication on animal growth and productivity (Golian et al., 2010). In the current study, the plasma IgM and IgG were higher (P<0.05) for chickens fed low CP diets supplemented with amino acids (Figure 1). L-threonine was added to broiler diets to balance the unique nutritional requirements of the animals and served as a component to body protein that is involved in immune responses of the animals (Lemme, 2003; Ayasan et al., 2009). Golian et al. (2010) reported that low dietary CP was not significantly different in anti-SRBC, IgG, and IgM antibody titers as compared with high-protein diet. Takahashi et al. (1995) reported that plasma acute-phase of α1-acid glycoprotein concentration and interleukin-1-like activity after

12

Gut microflora and intestinal morphology changes of broiler chickens fed reducing dietary protein...
Shazali et al.

Escherichia coli lipopolysaccharide injection in chicks fed the low-protein diet was greater than those of fed the high-protein diet.

Conclusions

Broilers fed a low crude protein diet supplemented with amino acids have improved growth performance, reduced *Enterobacteriaceae*, increased lactic acid bacteria counts, and increased small intestine villi height and crypt depth, as well as ileal digesta volatile fatty acid concentrations. However, reducing dietary crude protein up to 2% shows the best results, especially in growth performance, feed conversion ratio, *Enterobacteriaceae* count, duodenal and jejunal villi heights, ileal digesta volatile fatty acid concentrations, and immunity status. It is believed that by reducing the level of crude protein in the broiler diet while supplementing it with commercial amino acids may enhance the nutrient digestibility and absorption in broiler chickens, and simultaneously will give better performance.

Acknowledgments

References

Abbasi, M. A.; Mahdavi, A. H.; Samie, A. H. and Jahanian, R. 2014. Effects of different levels of dietary crude protein and threonine on performance, humoral immune responses and intestinal morphology of broiler chicks. Brazilian Journal of Poultry Science 16:35-44. https://doi.org/10.1590/S1516-635X2014000100005

Abeyesinghe, S. M.; Wathes, C. M.; Nicol, C. J. and Randall, J. M. 2001. The aversion of broiler chickens to concurrent vibrational and thermal stressors. Applied Animal Behaviour Science 73:199-215. https://doi.org/10.1016/S0168-1591(01)00142-3

Aletor, V. A.; Hamid, I. I.; Nieß, E. and Pfeffer, E. 2000. Low-protein amino acid supplemented diets in broilers chickens: effects on performance, carcass characteristics, whole-body composition and efficiencies of nutrient utilisation. Journal of Science Food Agriculture 80:547-554. https://doi.org/10.1002/(SICI)1097-0010(200004)80:5%3C547::AID-JSFA531%3E3.0.CO;2-C

Attia, Y. A.; El-Tahawy, W. S.; Abd El-Hamid, A. E. H. E.; Hassan, S. S.; Nizza, A. and El-Kelaway, M. I. 2012. Effect of phytase with or without multienzyme supplementation on performance and nutrient digestibility of young broiler chicks fed mash or crumble diets. Italian Journal of Animal Science 11:e56. https://doi.org/10.4081/ijas.2012.e56

Ayasan, T. and Okan, F. 2010. Effects of diets containing different levels of threonine and lysine amino acids on fattening performance of broiler chicks. Journal of Faculty Agriculture Suleyman Demirel University 5:36-43.

Ayasan, T.; Okan, F. and Hizli, H. 2009. Threonine requirement of broiler from 22-42 days. International Journal of Poultry Science 8:862-865. https://doi.org/10.3923/ijps.2009.862.865

Bailey, J. S. 1988. Integrated colonization control of *Salmonella* in poultry. Poultry Science 67:928-932. https://doi.org/10.3382/ps.0670928

Bailey, M. T. and Coe, C. L. 1999. Maternal separation disrupts the integrity of the intestinal microflora in infant rhesus monkeys. Developmental Psychobiology 35:146-155.

Ball, R. O. 2001. Threonine requirement and the interaction between threonine intake and gut mucins in pigs. Symposium of the 2001 Degussa; Banff Pork Seminar. Banff, Alberta, Canada.

Banerjee, S.; Melesse, A.; Dotamo, E.; Berihun, K. and Beyan, M. 2013. Effect of feeding different dietary protein levels with iso-caloric ration on nutrients intake and growth performances of dual-purpose koekoeck chicken breeds. International Journal of Applied Poultry Research 2:27-32.

Baylan, M.; Canogullari, S.; Ayasan, T. and Sahin, A. 2006. Dietary threonine supplementation for improving growth performance and edible carcass parts in Japanese quails, *Coturnix coturnix Japonica*. International Journal of Poultry Science 5:635-638. https://doi.org/10.3923/ijps.2006.635.638

Bikker, P.; Dirkzwager, A.; Fledderus, J.; Trevisi, P.; Huërou-Luron, I. le; Lallès, J. P. and Awati, A. 2006. The effect of dietary protein and fermentable carbohydrates levels on growth performance and intestinal characteristics in newly weaned piglets. Journal of Animal Science 84:3337-3345. https://doi.org/10.2527/jas.2006-076

Bowmaker, J. E. and Gous, R. M. 1991. The response of broiler breeder hens to dietary lysine and methionine. British Poultry Science 32:1069-1088. https://doi.org/10.1080/00071669108417430

Bregendahl, K.; Sell, J. L. and Zimmerman, D. R. 2002. Effect of low-protein diets on growth performance and body composition of broiler chicks. Poultry Science 81:1156-1167. https://doi.org/10.1093/ps/81.8.1156

Burkholder, K. M.; Thompson, K. L.; Einstein, M. E.; Applegate, T. J. and Patterson, J. A. 2008. Influence of stressors on normal intestinal microbiota, intestinal morphology, and susceptibility to *Salmonella Enteritidis* colonization in broilers. Poultry Science 87:1734-1741. https://doi.org/10.3382/ps.2008-00107

Cherian, G.; Orr, A.; Burke, I. C. and Pan, W. 2013. Feeding *Artemisia annua* alters digesta pH and muscle lipid oxidation products in broiler chickens. Poultry Science 92:1085-1090. https://doi.org/10.3382/ps.2012-02752

Choe, D. W.; Loh, T. C.; Foo, H. L.; Hair-Bejo, M. and Awis, Q. S. 2012. Egg production, faecal pH and microbial population, small intestine morphology, and plasma and yolk cholesterol in laying hens given liquid metabolites produced by Lactobacillus plantarum strains. British Poultry Science 53:106-115. https://doi.org/10.1080/00071668.2012.659653

Cobb-Vantress. 2013. Management guides. Available at: <http://www.cobb-vantress.com/academy/management-guides>. Accessed on: Oct. 1, 2013.

Corzo, A.; Fritts, C. A.; Kidd, M. T. and Kerr, B. J. 2005. Response of broiler chicks to essential and non-essential amino acid supplementation of low crude protein diets. Animal Feed Science and Technology 118:319-327. https://doi.org/10.1016/j.anifeedsci.2004.11.007

Corzo, A.; Moran, E. T. Jr and Hoohler, D. 2002. Lysine need of heavy broiler males applying the ideal protein concept. Poultry Science 81:1863-1868. https://doi.org/10.1093/ps/81.12.1863

Dari, R. L.; Penz Jr., A. M.; Kessler A. M. and Jost H. C. 2005. Use of digestible amino acids and the concept of ideal protein in feed formulation for broilers. Journal of Applied Poultry Research 14:195-203. https://doi.org/10.1093/japr/14.2.195

de Beer, M. 2011. Current trends in broiler breeder nutrition. In: Proceedings of the III International Symposium on Nutritional Requirements Poultry and Swine, Viçosa, MG, Brasil.

Foo, H. L.; Loh, T. C.; Law, F. L.; Lim, Y. Z.; Kufli, C. N. and Rusul, G. 2003. Effects of feeding *Lactobacillus plantarum* I-UL4 isolated from Malaysian tempeh on growth performance, faecal flora and lactic acid bacteria and plasma cholesterol concentrations in postweaning rats. Food Science and Biotechnology 4:403-408.

Franklin, M. A.; Mathew, A. G.; Vickers, J. R. and Clift, R. A. 2002. Characterization of microbial populations and volatile fatty acid concentrations in the jejunum, ileum, and cecum of pigs weaned at 17 vs. 24 days of age. Journal of Animal Science 80:2904-2910. https://doi.org/10.2527/2002.80112904x

Furlan, R. L.; Faria Filho, D. E.; Rosa, P. S. and Macari, M. 2004. Does low-protein diet improve broiler performance under heat stress conditions? Brazilian Journal of Poultry Science 6:71-79. https://doi.org/10.1590/S1516-635X2004000200001

Golian, A.; AamiAzghadi, M. and Pilevar, M. 2010. Influence of various of energy and protein on performance and humoral immune responses in broiler chick. Global Veterinarian 4:434-440.

Gomide, E. M.; Rodrigues, P. B.; Naves, L. P.; Bernardino, V. M. P.; Santos, L. M. and Garcia, A. A. P. 2012. Diets with reduced levels of nutrients supplemented with phytase and amino acids for broilers. Ciência e Agrotecnologia 36:100-107. https://doi.org/10.1590/S1413-70542012000100013

Gu, X. and Li, D. 2004. Effect of dietary crude protein level on villous morphology, immune status and histochemistry parameters of digestive tract in weaning piglets. Animal Feed Science and Technology 114:113-126. https://doi.org/10.1016/j.anifeedsci.2003.12.008

Han, I. K. and Lee, J. H. 2000. The role of synthetic amino acids in monogastric animal production. Asian-Australasian Journal of Animal Sciences 13:543-560. https://doi.org/10.5713/ajas.2000.543

Htoo, J. K.; Araiza, B. A.; Sauer, W. C.; Rademacher, M.; Zhang, Y.; Cervantes, M. and Zijlstra, R. T. 2007. Effect of dietary protein content on ileal amino acid digestibility, growth performance, and formation of microbial metabolites in ileal and caecal digesta of early-weaned pigs. Journal of Animal Science 85:3303-3312. https://doi.org/10.2527/jas.2007-0105

Humphrey, T. 2006. Are happy chickens safer chickens? Poultry welfare and disease susceptibility. British Poultry Science 47:379-391. https://doi.org/10.1080/00071660600829084

Incharoen, T. and Yamauchi, K. 2009. Production performance, egg quality and intestinal histology in laying hens fed dietary dried fermented ginger. International Journal of Poultry Science 8:1078-1085. https://doi.org/10.3923/ijps.2009.1078.1085

Incharoen, T.; Khambualai, O. and Yamauchi, K. 2009. Performance and histological changes of the intestinal villi in chickens fed dietary natural zeolite including plant extract. Asian Journal of Poultry Science 3:42-50. https://doi.org/10.3923/ajpsaj.2009.42.50

Isaacson, R. E.; Firkins, L. D.; Weigel, R. M.; Zuckermann, F. A. and Di Petrio J. A. 1999. Effect of transportation and feed withdrawal on shedding of *Salmonella typhimurium* among experimentally infected pigs. American Journal in Veterinary Research 60:1155-1158.

14

Gut microflora and intestinal morphology changes of broiler chickens fed reducing dietary protein...
Shazali et al.

Jiang, Q.; Waldroup, P. W. and Fritts, C. A. 2005. Improving the utilization of diets low in crude protein for broiler chicken 1. Evaluation of special amino acid supplementation to diets low in crude protein. International Journal of Poultry Science 4:115-122. https://doi.org/10.3923/ijps.2005.115.122

Kamran, Z.; Mirza, M. A.; Haq, A. U. and Mahmood, S. 2004. Effect of decreasing dietary protein levels with optimum amino acids profile on the performance of broilers. Pakistan Veterinary Journal 24:165-168.

Kidd, M. T.; Lerner, S. P.; Allard, J. P.; Rao, S. K. and Halley, J. T. 1999. Threonine needs of finishing broilers: growth, carcass and economic responses. Journal of Applied Poultry Research 8:160-169. https://doi.org/10.1093/japr/8.2.160

Labadan, M. C. Jr.; Hsu, K. N. and Austic, R. E. 2001. Lysine and arginine requirements of broiler chickens at two to three week intervals to eight weeks of age. Poultry Science 80:599-606. https://doi.org/10.1093/ps/80.5.599

Laudadio, V.; Dambrosio, A.; Normanno, G.; Khan, R. U.; Naz, S.; Rowghani, E. and Tufarelli, V. 2012. Effect of reducing dietary protein level on performance responses and some microbiological aspects of broiler chickens under summer environmental conditions. Avian Biology Research 5:88-92. https://doi.org/10.3184/175815512X13350180713553

Law, G.; Adjiri-Awere, A.; Pencharz, P. B. and Ball, R. O. 2000. Gut mucus in piglets are dependent upon dietary threonine. Advances in Pork Production. Proceedings of the 2000 Banff Pork Seminar 11:10.

Lemme, A. 2003. Reassessing amino acid levels for Pekin ducks. Poultry International Journal 42:18-24.

Loh, T. C.; Choe, D. W.; Foo, H. L.; Sazili, A. Q. and Bejo, M. H. 2014. Effects of feeding different postbiotic metabolite combinations produced by *Lactobacillus plantarum* strains on egg quality and production performance, faecal parameters and plasma cholesterol in laying hens. BMC Veterinary Research 10:149. https://doi.org/10.1186/1746-6148-10-149

Loh, T. C.; Foo, H. L.; Tan, S. H.; Goh, Y. M.; Shukriyah, M. H. and Kufli, C. N. 2003. Effects of fermented products on performance, faecal pH, *Enterobacteriaceae* and lactic acid bacteria counts and interrelationships and plasma cholesterol concentration in rats. Journal Animal Feed Science 12:633-644. https://doi.org/10.22358/jafs/67757/2003

Loh, T. C.; Law, F. L.; Goh, Y. M.; Foo, H. L. and Zulkifli, I. 2009. Effects of feeding fermented fish on egg cholesterol content in hens. Animal Science Journal 80:27-33. https://doi.org/10.1111/j.1740-0929.2008.00591.x

Lopez, G. and Leeson, S. 1994. Egg weight and offspring performance of older broiler breeders fed low-protein diets. Journal of Applied Poultry Research 3:164-170. https://doi.org/10.1093/japr/3.2.164

Meddings, J. B. and Swain, M. G. 2000. Environmental stress induced a generalized increase in gastrointestinal permeability mediated by endogenous glucocorticoids in the rat. Gastroenterology 119:1019-1028.

Mountzouris, K. C.; Tsistsikos, P.; Kalamara, E.; Nitsh, S.; Schatzmayr, G. and Fegeros, K. 2007. Evolution of the efficiency of a probiotic containing *Lactobassilus, Bifidobacterium, Enterococcus,* and *Pediococcus* strains in promoting broiler performance and modulating ceacal microflora composition and metabolic activities. Poultry Science 86:309-317. https://doi.org/10.1093/ps/86.2.309

Mulder, R. W. A. W. 1995. Impact of transport and related stresses on the incidence and extent of human pathogens in pig meat and poultry. Journal of Food Safety 15:239-246. https://doi.org/10.1111/j.1745-4565.1995.tb00136.x

Moran Jr., E. T. 2007. Nutrition of the developing embryo and hatchling. Poultry Science 86:1043-1049. https://doi.org/10.1093/ps/86.5.1043

Namroud, N. F.; Shivazad, M. and Zaghari, M. 2008. Effects of fortifying low crude protein diet with crystalline amino acids on performance, blood ammonia level, and excreta characteristics of broiler chicks. Poultry Science 87:2250-2258. https://doi.org/10.3382/ps.2007-00499

Nyachoti, C. M.; Omogbenigun, F. O.; Rademacher, M. and Blank, G. 2006. Performance responses and indicators of gastrointestinal health in early-weaned pigs fed low-protein amino acid-supplemented diets. Journal of Animal Science 84:125-134. https://doi.org/10.2527/2006.841125x

Oliveira, W. P.; Oliveira, R. F. M.; Donzele, J. L.; Albino, L. F. T.; Campos, P. H. R. F.; Balbino, E. M.; Maia, A. P. A. and Pastore, S. M. 2013. Lysine levels in diets for broilers from 8 to 21 days of age. Revista Brasileira de Zootecnia 42:869-878. https://doi.org/10.1590/S1516-35982013001200006

Pluske, J. R.; Black, B.; Pethick, D. W.; Mullan, B. P. and Hampson, D. J. 2003. Effects of different sources of dietary fiber in diets on performance, digesta characteristics and antibiotic treatment of pigs after weaning. Animal Feed Science and Technology 107:129-142. https://doi.org/10.1016/S0377-8401(03)00072-5

Rasekh, J.; Thaler, A. M.; Engeljohn, D. L. and Pihkala, N. H. 2005. Food safety and inspection service policy for control of poultry contaminated by digestive tract contents: a review. Journal of Applied Poultry Research 14:603-611. https://doi.org/10.1093/japr/14.3.603

Rezaeipour, V. and Gazani, S. 2014. Effects of feed form and feed particle size with dietary L- threonine supplementation on performance, carcass characteristics and blood biochemical parameters of broiler chickens. Journal of Animal Science and Technology 56:20. https://doi.org/10.1186/2055-0391-56-20

Rigby, C. E. and Pettit, J. R. 1980. Changes in the *Salmonella* status of broiler chickens subjected to simulated shipping conditions. Canadian Journal of Comparative Medicine 44:374-381.

15

Gut microflora and intestinal morphology changes of broiler chickens fed reducing dietary protein...
Shazali et al.

Saima, M. Z. U. Khan; Jabbar, M. A.; Mehmud, A.; Abbas, M. M. and Mahmood, A. 2010. Effect of lysine supplementation in low protein diets on the performance of growing broilers. Pakistan Veterinary Journal 30:17-20.

Saunders, P. R.; Kosecka, U.; McKay, D. M. and Perdue, M. H. 1994. Acute stressors stimulate ion secretion and increase epithelial permeability in rat intestine. American Journal of Physiology 267:794-799. https://doi.org/10.1152/ajpgi.1994.267.5.G794

Schaart, M. W.; Schierbeek, H.; van der Schoor, S. R. D.; Stoll, B.; Burrin, D. G.; Reeds, P. J. and van Goudoever, J. B. 2005. Threonine utilization is high in the intestine of piglets. Journal of Nutrition 135:765-770. https://doi.org/10.1093/jn/135.4.765

Shazali, N.; Foo, H. L.; Loh, T. C.; Choe, D. W. and Rahim, R. A. 2014. Prevalence of antibiotic resistance in lactic acid bacteria isolated from the faeces of broiler chicken in Malaysia. Gut Pathogens 6:1. https://doi.org/10.1186/1757-4749-6-1

Soderholm, J. D.; Yates, D. A.; Gareau, M. G.; Yang, P. C.; MacQueen, G. and Perdue, M. H. 2002. Neonatal maternal separation predisposes adult rats to colonic barrier dysfunction in response to mild stress. American Journal Physiology 283:1257-1263. https://doi.org/10.1152/ajpgi.00314.2002

Sritiawthai, E.; Sakulthai, S.; Sakdee, J.; Bunchasak, C.; Kaewtapee, C. and Poeikhampha, T. 2013. Effect of protein level and dietary energy on production, intestinal morphology, and carcass yield of meat duck during starter phase of 14 days. Journal of Applied Sciences 13:315-320. https://doi.org/10.3923/jas.2013.315.320

St-Pierre, N. R.; Cobanov, B. and Schnitkey, G. 2003. Economic losses from heat stress by US livestock industries. Journal of Dairy Science 86:52-77. https://doi.org/10.3168/jds.S0022-0302(03)74040-5

Swatson, H. K.; Gous, R.; Iji, P. A. and Zarrinkalam, R. 2002. Effect of dietary protein level, amino acid balance and feeding level on growth, gastrointestinal tract, and mucosal structure of the small intestine in broiler chickens. Animal Research 51:501-515. https://doi.org/10.1051/animres:2002038

Takahashi, K.; Yodogawa, S.; Akiba, Y. and Tamura, K. 1995. Effect of dietary protein concentration on responses to *Escherichia coli* endotoxin in broiler chickens. British Journal of Nutrition 74:173-182. https://doi.org/10.1079/bjn19950121

Tannock, G. W. and Savage, D. C. 1974. Influences of dietary and environmental stress on microbial populations in the murine gastrointestinal tract. Infection and Immunity 9:591-598.

Tenesa, M.; Loh, T. C.; Foo, H. L.; Samsudin, A. A.; Mohamad, R. and Raha, A. R. 2016. Effects of feeding different levels of low crude protein diets with different levels of amino acids supplementation on layer hen performance. Pertanika Journal Tropical Agriculture Science 39:543-555.

Tsukahara, T.; Koyama, H.; Okada, M. and Ushida, K. 2002. Stimulation of butyrate production by gluconic acid in batch culture of pig cecal digesta and identification of butyrate-producing bacteria. Journal of Nutrition 132:2229-2234. https://doi.org/10.1093/jn/132.8.2229

Valizade, M. R.; Sadeghi, A. A.; Chamani, M.; Shawrang, P. and Kashan, N. 2016. The effects of increase in threonine to lysine ratio on performance, blood parameters and humoral immune responses of male broiler chickens challenged with *Salmonella*. Kafkas Üniversitesi Veteriner Fakültesi Dergisi 22:165-172.

Waldroup, P. W.; Jiang, Q. and Fritts, C. A. 2005. Effects of supplementing broiler diets low in crude protein with essential and nonessential amino acids. International Journal of Poultry Science 4:425-431. https://doi.org/10.3923/ijps.2005.425.431

Whiley, H.; Akker, B.; Giglio, S. and Bentham, R. 2013. The role of environmental reservoirs in human *Campylobacteriosis.* International Journal of Environmental Research and Public Health 10:5886-5907. https://doi.org/10.3390/ijerph10115886

Wilson, H. R. 1997. Effects of maternal nutrition on hatchability. Poultry Science 76:134-143. https://doi.org/10.1093/ps/76.1.134

Wu, G. 1998. Intestinal mucosal amino acid catabolism. Journal of Nutrition 128:1249-1252. https://doi.org/10.1093/jn/128.8.1249

Wu, G.; Meier, S. A. and Knabe D. A. 1996. Dietary glutamine supplementation prevents jejunal atrophy in weaned pigs. Journal of Nutrition 126:2578-2584. https://doi.org/10.1093/jn/126.10.2578

Zhai, W.; Peebles, E. D.; Wang, X.; Gerard, P. D.; Olanrewaju, H. A. and Mercier, Y. 2016a. Effects of dietary lysine and methionine supplementation on Ross 708 male broilers from 21 to 42 d of age (III): serum metabolites, hormones, and their relationship with growth performance. Journal of Applied Poultry Research 25:223-231. https://doi.org/10.3382/japr/pfw004

Zhai, W.; Peebles, E. D.; Schilling, M. W. and Mercier, Y. 2016b. Effects of dietary lysine and methionine supplementation on Ross 708 male broilers from 21 to 42 d of age (I): growth performance, meat yield, and cost effectiveness. Journal of Applied Poultry Research 25:197-211. https://doi.org/10.3382/japr/pfw002