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Small Intestine Characteristics and Nutrient Retention in Broiler Chickens Submitted to Different Protein Regimes and Betaine Supplementation

Adi Ratriyanto* and Sunarto

Department of Animal Science, Faculty of Agriculture, Universitas Sebelas Maret, Surakarta, 57126, Indonesia

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* Corresponding author:
E-mail: ratriyanto@staff.uns.ac.id

ABSTRACT

This study was designed to determine the intestinal characteristics and nutrient retention of broiler chickens subjected to different protein regimes supplemented with betaine. Four experimental diets were formulated, consisting of two basal diets containing 20% and 23% crude protein (CP), both with and without betaine supplementation at the level of 0.14%. The diets were applied to 180 broiler chickens that were randomly allotted to 2×2 factorial arrangement with five replicates of nine chickens each. The diet with 20.0% CP generated better small intestine characteristics than the diet with 23.0% CP as indicated by the longer ileum and total small intestine length ($P<0.05$). This improvement was associated with lessened CP excretion and improved dry matter (DM) and CP retention ($P<0.05$) in the birds fed 20% CP. Furthermore, dietary betaine supplementation enhanced the ileum, total small intestine length, villus height, and villus-to-crypt ratio ($P<0.05$), indicating a greater surface area for nutrient absorption. This enhancement was reflected in the reduction of DM and CP excretion and improvement in DM and CP retention in the betaine supplemented group ($P<0.05$). Therefore, we conclude that feeding a diet of 20% CP with betaine supplementation improved the small intestine characteristics and nutrient retention of broiler chickens.

Keywords: Betaine, Broilers, Environmental temperature, Nutrient absorption, Nutrient excretion

Introduction

The development of the digestive tract is an important factor that determines the growth performance and health of broiler chickens (Esmailzadeh *et al*., 2016) and affects the microbial population, which has important role in nutrient digestion and the immune system (Esmailzadeh *et al*., 2016; Ratriyanto and Mosenthin, 2018). Furthermore, the dietary protein level has an impact on the growth rate of the chickens (De Faria Filho *et al*., 2007; Moosavi *et al*., 2012). However, high environmental temperatures in tropical areas such as in Indonesia may obstruct the growth performance of broilers by limiting protein synthesis (Rashid *et al*., 2012). Several studies have shown that high environmental temperatures reduce protein synthesis, but feeding them with a higher protein level cannot compensate for this effect (Moosavi *et al*., 2012; Rashid *et al*., 2012). Other studies discovered that feeding broiler chickens with higher protein levels in a high-temperature environment suppressed their growth rate (Moosavi *et al.,* 2012; Gous *et al*., 2018), which could be attributed to their increased heat production (Daghir, 2009). Feeding them with a high protein level also increased nitrogen excretion from their bodies, indicating an inefficient use of nutrients (De Faria Filho *et al*., 2007; Moosavi *et al*., 2012). Therefore, it is necessary to provide a precise protein level for broiler chickens in tropical areas.

The use of betaine (trimethyl glycine) in poultry diet has increased in the last decades and commonly has aimed to improve the growth performance and carcass characteristics (Li *et al*., 2011; Rao *et al*., 2011). Betaine is a methyl group (CH_3) donor and is involved in protein and energy metabolism (Ratriyanto and Mosenthin, 2018). Dietary betaine can increase methionine availability, which leads to an improvement in protein availability (Ratriyanto *et al*., 2009; Rao *et al*., 2011). This function of betaine can improve protein efficiency through supplementing with betaine and reducing the protein level in the diet (Li *et al*., 2011; Ratriyanto *et al*., 2014). Betaine also acts as an organic osmolyte by stabilizing intestinal epithelial cells and supporting intestinal microbial growth (Metzler-Zebeli *et al*., 2009; Weiss *et al*., 2013), which are reflected in the improvement of nutrient retention and the growth performance of broiler chickens (El-Husseiny *et al*., 2007; Chand *et al*., 2017).

Many studies have shown the positive effects of dietary betaine supplementation in poultry as indicated by the improvement in growth performances and carcass quality (Rao *et al*., 2011; Sakomura *et al*., 2013; Liu *et al*., 2019). Currently, there are only a small number of studies focusing on the impacts of dietary betaine on the intestinal characteristics and nutrient retention of broiler chickens and the responses observed in these studies vary (Sayed and Downing, 2011; Sakomura *et al*., 2013). Therefore, the objective of this study was to determine the intestinal characteristics and nutrient retention of broiler chickens submitted to different protein regimes supplemented with betaine.

Materials and Methods

Animals and diets

In total, 180 one-day-old unsexed Lohmann broiler chickens were randomly allocated into the four dietary treatments with five replicates of nine chickens each. The arrangement of the treatments corresponds to a 2×2 factorial design with two levels of crude protein (CP, 20 and 23%) and two levels of betaine supplementation (0% and 0.14%).

The basal diet was formulated according to the standard of the National Research Council (1994) except for the protein levels of 20% and 23%, and each protein level contained the same amount of metabolizable energy, namely 3,200 kcal/kg (Table 1). Each protein level was fed without supplementation (Control) or supplemented with 0.14% betaine (Betaine). The supplementation of betaine to the basal diets was performed at the expense of corn, following the procedure used by Ratriyanto *et al*. (2014).

Determination of intestinal characteristics

The chickens were reared in 20 floor pens (1×1 m) under natural temperature conditions until 35 days of age. The average ambient temperature at the midday during the experiment was 32.9ºC. Water and feed were supplied *ad libitum*. On day 35, two chickens per pen (40 chickens in total) were randomly selected and slaughtered after fasting for 12 hours to measure their intestinal characteristics. The small intestine was removed from each bird and the lengths of the duodenum, jejunum, and ileum were measured using a gauge. The villus height and crypt depth were determined using a 1 cm section of distal jejunum following the procedure of Kettunen *et al*. (2001a). The sections were fixed in a 4% paraformaldehyde solution and dehydrated thereafter. The samples were set into paraffin blocks, cut at 5 µm, and stained with hematoxylin-eosin. A cross-section of each sample was captured at 40× magnification with a digital camera attached to a microscope. Villus height was measured from the base to the top of the villi and crypt depth was measured from the base of the villi to the base of the crypt as presented in Figure 1 (Dos Santos *et al.*, 2018). The villus/crypt ratio was calculated as villus height divided by the mucosal crypt depth in each preparation (Kettunen *et al*., 2001a).

Table 1. Composition (%) and nutrient content of experimental diets

Ingredients	CP 20%		CP 23%	
	Control	Betaine	Control	Betaine
Yellow corn	56.75	56.61	55.00	54.86
Rice bran	10.35	10.35	4.93	4.93
Soybean meal	20.60	20.60	25.00	25.00
Fishmeal	7.00	7.00	10.00	10.00
Coconut oil	3.60	3.60	3.95	3.95
L-lysine HCl	0.08	0.08	0.00	0.00
DL-methionine	0.11	0.11	0.07	0.07
Dicalcium phosphate	0.50	0.50	0.10	0.10
Limestone	0.56	0.56	0.50	0.50
Premix*	0.20	0.20	0.20	0.20
NaCl	0.25	0.25	0.25	0.25
Betaine	-	0.14	-	0.14
Nutrient content				
Crude protein (%)	20.00	19.99	23.00	22.98
Metabolizable energy (kcal/kg)	3,200	3,195	3,200	3,195
Crude fiber (%)	7.07	7.06	6.66	6.65
Methionine (%)	0.50	0.50	0.51	0.51
Lysine (%)	1.18	1.18	1.20	1.20
Calcium (%)	1.01	1.01	1.01	1.01
Available phosphorus (%)	0.48	0.48	0.48	0.48

* The premix supplied the following per kilogram diets: 12,000 IU vitamin A; 2,400 IU vitamin D; 5 mg vitamin E; 6 mg vitamin K; 4 mg vitamin B1; 6 mg vitamin B2; 2 mg vitamin B6; 4 mg vitamin B12; 28 mg vitamin C; 30 mg nicotinic acid; 10 mg calcium D-pantothenate; 150 mg electrolytes containing Na, K, Ca, and Mg.

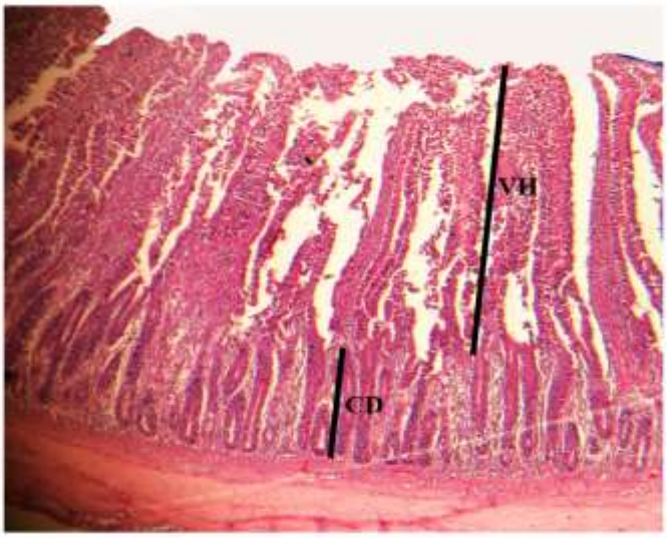

Figure 1. Example of jejunal villus height (VH) and crypt depth (CD) determination in 20% CP supplemented with betaine.

Determination of nutrient retention

After 35 days of dietary treatments, 40 chickens (two per replicate) were randomly selected for a digestion trial to measure nutrient retention for each experimental diet. The chickens were housed in individual cages and fed the tested diet for a five-day collection period according the procedure used by El-Husseiny *et al*. (2007). The chickens were given an experimental diet containing 0.4% Fe_2O_3 as an indicator to determine the start and the end of excreta collection (Marais, 2000). At the end of collection period, the chickens were fed diets without the indicator. Excreta collection was started when the red color appeared in the excreta and was terminated when the red color of the excreta disappeared (Ratriyanto *et al*., 2014). During the excreta collection, 2 ml 0.2 N H_2SO_4 was spread periodically on the excreta to minimize further bacterial fermentation. The excreta were pooled and dried under the sun thereafter. Samples of the diets and excreta were milled through a 1.0 mm mesh screen prior to analysis. The determination of the dry matter (DM) and CP was performed as outlined by Association of Official Analytical Chemists (AOAC, 2001).

Statistical analysis

The data were submitted to a two-way analysis of variance with the following model: $y_{ijk} = \mu + \alpha_i + \beta_j + \alpha\beta_{ij} + \varepsilon_{ijk}$, where μ = the general mean, α_i = the effect of protein levels, β_j = the effect of betaine supplementation, $\alpha\beta_{ij}$ = the interaction effect of protein levels and betaine supplementation, and ε_{ijk} = an experimental error. If the variance analysis results indicated a significant effect, it was then subjected to Duncan's Multiple Range Test at P<0.05 (Steel *et al*., 1996). All statistical analyses were performed using the R program, version 3.5.3 (R Core Team, 2019).

Results and Discussion

Small intestine characteristics

No interaction occurred between the protein level and betaine supplementation on small intestine characteristics (Table 2), which indicates that these values remained the same for all protein levels, with or without betaine supplementation. The birds fed 20% CP exhibited a longer ileum (78.77 cm vs 73.88 cm, P<0.01), which corresponded to a longer small intestine (199.61 cm vs 192.31 cm, P=0.04). Furthermore, betaine supplementation significantly increased the ileum (78.27 cm vs 74.38 cm, P=0.02) and small intestine length (199.95 cm vs 191.97 cm, P=0.03) as well as the villus height (376 µm vs 318 µm, P<0.01) and villus height/crypt depth ratio (4.83 vs 4.01, P=0.03) compared to that of a non-supplemented diet, indicating more gut surface area for nutrient absorption. This finding is confirmed by the higher nutrient retention in this group (Tables 3 and 4). These results are comparable with observation in broilers where the jejunal villus height ranged between 318-397 µm, crypt depth ranged between 60-68 µm, and villus height/crypt depth ratio ranged between 3.27-8.25 (Liu *et al*., 2019).

This research confirms that intestinal morphology was improved by betaine supplementation. Betaine supplementation stabilizes the mucosal structure in birds due to its osmolytic property, which is indicated by a longer small intestine, a higher villus, and a higher villus/crypt ratio (Table 2). This finding agrees with previous observation, in which betaine increased the villus/crypt ratio and small intestine length in broilers aged 21 days, indicating that betaine altered the intestinal cells' morphology and stabilized the gut mucosa structure (Kettunen *et al*., 2001b; Kettunen *et al*., 2001c). Other observations of broiler chickens also revealed that

Table 2. Small intestine characteristics in broilers submitted to different protein regimes supplemented with betaine

Treatments		Duodenum length (cm)	Jejunum length (cm)	Ileum length (cm)	Small Intestine length (cm)	Villus Height (µm)	Crypt Depth (µm)	Villus/Crypt Ratio
Interaction protein × betaine								
20	Control	36.25 ± 2.10	83.31 ± 4.91	77.38 ± 4.89	196.94 ± 9.96	309 ± 41	79 ± 9.7	3.91 ± 0.05
20	Betaine	37.00 ± 2.83	85.13 ± 1.18	80.16 ± 2.09	202.28 ± 5.22	352 ± 36	71 ± 3.3	4.96 ± 0.70
23	Control	33.75 ± 0.87	81.88 ± 5.48	71.38 ± 1.75	187.00 ± 6.12	327 ± 16	80 ± 9.0	4.10 ± 0.33
23	Betaine	37.50 ± 3.49	83.75 ± 4.91	76.38 ± 1.80	197.63 ± 0.75	400 ± 25	85 ± 4.4	4.69 ± 0.51
P value		0.26	0.98	0.46	0.43	0.40	0.28	0.77
Effect of protein								
20		36.63 ± 2.34	84.22 ± 3.45	78.77 ± 3.78[a]	199.61 ± 7.90[a]	330 ± 42	75 ± 7.7	4.43 ± 0.73
23		35.63 ± 3.09	82.81 ± 4.92	73.88 ± 3.14[b]	192.31 ± 6.97[b]	363 ± 44	83 ± 7.0	4.40 ± 0.50
P value		0.44	0.54	<0.01	0.04	0.07	0.20	0.40
Effect of betaine								
Control		35.00 ± 2.00	82.59 ± 4.88	74.38 ± 4.67[b]	191.97 ± 9.32[b]	318 ± 30[b]	80 ± 8.4	4.01 ± 0.24[a]
Betaine		37.25 ± 2.95	84.44 ± 3.39	78.27 ± 2.71[a]	199.95 ± 4.26[a]	376 ± 38[a]	78 ± 8.5	4.83 ± 0.57[b]
P value		0.10	0.43	0.02	0.03	<0.01	0.81	0.03

[a,b] Mean in the same column and factor followed with different superscripts indicated significant difference (P<0.05).

supplemental betaine enhanced the villus height and villus height/crypt depth ratio in the duodenum and jejunum (Liu *et al*., 2019). In another study, betaine increased the villus height associated with the absorptive area in broilers, although the small intestine length was not affected (Dos Santos *et al*., 2018). Increasing the villus height supports better nutrient digestion and absorption, leading to an improvement in growth performance in the long term (Dos Santos *et al*., 2018). In contrast to this result, Sakomura *et al*. (2013) did not observe any alteration in villus height or crypt depth in broilers receiving betaine supplementation.

Several of betaine's modes of action may explain the positive response in intestinal characteristics to supplemental betaine. As a methyl donor, betaine improves protein and energy metabolism, leading to an improvement in the intestinal cells' proliferation as indicated by the longer small intestine and higher villus in this study (Ratriyanto and Mosenthin, 2018; Liu *et al*., 2019). Furthermore, the osmolytic function of betaine helps the intestine cope with the high osmotic pressure created by unabsorbed nutrients in the lumen intestine, thus supporting the intestine's development (Ratriyanto and Mosenthin, 2018; Dos Santos *et al*., 2018; Ratriyanto and Prastowo, 2019). Betaine also lessens oxidative damage to the intestine due to its antioxidant activity (Liu *et al*., 2019).

Dry matter and crude protein retention

No interaction occurred between CP levels and betaine supplementation on DM and CP retention (Tables 3 and 4), which indicates that nutrient retention remained the same for the two CP levels, with or without betaine supplementation. The CP levels did not affect DM intake, excretion, or grams of retention. However, birds fed 20% CP levels generated a higher percentage of DM retention relative to DM intake (77.92% vs 75.88%, P=0.04), indicating a more efficient nutrient digestion and absorption (De Faria Filho *et al*., 2007). Improvement in the percentage of DM retention is associated with an improvement in the small intestine length in this study (Table 2), indicating a higher surface area for nutrient absorption.

Furthermore, compared with that of a non-supplemented diet, betaine supplementation reduced DM excretion (24.68 g vs 27.87 g, P=0.01) without affecting DM intake, leading to an increase in DM retention both in grams (89.89 g vs 85.23 g, P=0.04) and by percentage (78.45% vs 75.35%, P<0.01). These findings indicated that betaine yielded a better use of nutrients than a non-supplemented diet. Improvement in DM retention due to betaine supplementation could be attributed to the osmolytic property of betaine, which improved the intestinal cells' structure and the growth of intestinal microbiota, leading to enhanced nutrient digestion and absorption (Kettunen *et al*., 2001a; Ratriyanto and Prastowo, 2019). Dietary betaine also alleviated the digesta osmolality in the intestine, enabling higher nutrient absorption (Hamidi *et al*., 2010). Similar to this result, betaine addition enhanced the percentage of organic matter (OM) or DM retention in broiler chickens (El-Husseiny *et al*., 2007) and quails (Ratriyanto and Prastowo, 2019). Yet another observation of broiler chickens did not show any influence by betaine in the percentage of DM retention (Attia *et al*., 2005).

In contrast with DM intake, feeding a diet of 20% CP yielded a lower CP intake compared with feeding 23% CP (22.76 g vs 26.19 g, P<0.01). Feeding 20% CP also reduced the grams of CP excretion compared with feeding 23% CP (4.19 g vs 5.78 g, P<0.01). However, birds fed 20% CP generated a higher percentage of CP retention relative to CP intake (81.55% vs 77.93%, P<0.01). These results indicated that feeding a diet of 20% CP yielded a more efficient use of dietary CP. Feeding a high CP content leads to inefficiency due to high undigested and unabsorbed CP in the intestine, which is then excreted from the body (Moosavi *et al*., 2012). Moreover, feeding a high level of CP stimulates amino acid oxidation excesses and leads to low protein utilization (Blair *et al*., 1999). In accordance with this observation,

Table 3. Dry matter retention per bird per day in broilers submitted to different protein regimes supplemented with betaine

Treatments		Intake (g)	Excretion (g)	Retention (g)	Retention (%)
Interaction protein × betaine					
20	Control	113.02 ± 8.26	27.09 ± 3.26	85.93 ± 7.35	76.01 ± 2.63
20	Betaine	114.63 ± 6.03	23.11 ± 1.55	91.53 ± 5.49	79.82 ± 1.33
23	Control	113.18 ± 2.98	28.66 ± 2.74	84.53 ± 2.60	74.70 ± 2.12
23	Betaine	114.54 ± 1.84	26.28 ± 2.47	88.26 ± 1.60	77.07 ± 1.89
P value		0.96	0.49	0.67	0.44
Effect of protein					
20		113.82 ± 6.87	25.10 ± 3.19	88.73 ± 6.79	77.92 ± 2.81[a]
23		113.86 ± 2.44	27.47 ± 2.76	86.39 ± 2.83	75.88 ± 2.27[b]
P value		0.98	0.06	0.30	0.04
Effect of betaine					
Control		113.10 ± 5.85	27.87 ± 2.96[a]	85.23 ± 5.25[b]	75.35 ± 2.36[b]
Betaine		114.59 ± 4.20	24.69 ± 2.57[b]	89.89 ± 4.19[a]	78.45 ± 2.12[a]
P value		0.55	0.01	0.04	<0.01

[a,b] Mean in the same column and factor followed with different superscripts indicated significant difference (P<0.05).

Table 4. Crude protein retention per bird per day in broilers submitted to different protein regimes supplemented with betaine

Treatments		Intake (g)	Excretion (g)	Retention (g)	Retention (%)
Interaction protein × betaine					
20	Control	22.60 ± 1.65	4.42 ± 0.31	18.18 ± 1.74	80.35 ± 2.26
20	Betaine	22.93 ± 1.21	3.96 ± 0.76	18.96 ± 1.00	82.74 ± 2.93
23	Control	26.03 ± 0.69	6.06 ± 0.41	19.97 ± 0.46	76.72 ± 1.17
23	Betaine	26.34 ± 0.42	5.50 ± 0.48	20.85 ± 0.48	79.14 ± 1.72
P value		0.98	0.81	0.91	0.98
Effect of protein					
20		22.76 ± 1.37[b]	4.19 ± 0.60[b]	18.57 ± 1.40[b]	81.55 ± 2.77[a]
23		26.19 ± 0.56[a]	5.78 ± 0.52[a]	20.41 ± 0.64[a]	77.93 ± 1.89[b]
P value		<0.01	<0.01	<0.01	<0.01
Effect of betaine					
Control		24.32 ± 2.16	5.24 ± 0.93[a]	19.08 ± 1.53	78.53 ± 2.56[b]
Betaine		24.64 ± 1.99	4.73 ± 1.01[b]	19.90 ± 1.24	80.94 ± 2.95[a]
P value		0.53	0.04	0.09	0.02

[a,b] Mean in the same column and factor followed with different superscripts indicated significant difference (P<0.05)

previous studies showed that reducing dietary CP also reduced nitrogen excretion (Blair *et al*., 1999; De Faria Filho *et al*., 2007).

Betaine supplementation reduced CP excretion (4.73 g vs 5.24 g, P=0.04) without affecting CP intake compared to that of a non-supplemented diet and led to an increase in the percentage of CP retention (80.94% vs 78.53%, P=0.02) as presented in Table 4. Liu *et al*. (2019) observed an improvement in trypsin activity in the small intestine, which could be attributed to the enhanced CP retention. As an organic osmolyte, betaine adheres to the surface of biopolymers and helps proteins fold more compactly, leads to an improvement in protein digestion (Liu *et al*., 2019). The osmoprotective function of betaine also helps to maintain the intestinal cells' integrity, leading to enhanced nutrient absorption (Sakomura *et al*., 2013). Improvements in CP retention due to betaine supplementation have been observed previously in broiler chickens (El-Husseiny *et al*., 2007), laying hens (Attia *et al*., 2016), and ducks (Ahmed *et al*., 2018).

Conclusions

The findings of this experiment showed that diets with 20.0% CP generated better small intestine characteristics than diets with 23.0% CP as indicated by the longer ileum and total length of the small intestine. This improvement was associated with less CP excretion and improved

DM and CP retention. Furthermore, dietary betaine supplementation enhanced the ileum, total length of the small intestine, and villus-to-crypt ratio, indicating a greater surface area for nutrient absorption. This enhancement was reflected in the reduction of DM and CP excretion and the improvement in DM and CP retention.

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