

Encapsulated crystalline lysine and DL-methionine have higher efficiency than the crystalline form in broilers

Mingfa Sun, Hongchao Jiao, Xiaojuan Wang, Victoria A. Uyanga, Jingpeng Zhao,¹ and Hai Lin¹

Department of Animal Science, Shandong Agricultural University, Shandong Key Lab for Animal Biotechnology and Disease Control and Prevention, Taian City, Shandong Province, 271018, China

ABSTRACT Crystalline amino acids (AAs) exhibit high nutritional values when supplemented AA-deficient diets. However, the AAs in crystalline form in the diet are absorbed quickly than protein-bound AAs, which may take an effect on AA utilization efficiency. In this study, 2 experiments were conducted to investigate the effect of encapsulated lysine-HCl (Lys) and DL-methionine (DL-Met) on the growth performance of broiler chickens. In experiment 1, a total of 432 one-day-old male Arbor Acres broilers were subjected to 3 dietary treatments (27 pens; 16 birds per pen) for 42 d. The control group was basal diets supplemented with crystalline Lys and DL-Met, and treatment groups had basal diets supplemented with encapsulated Lys and DL-Met at the levels of 80% and 60% of control diets (80CLM, 60CLM), respectively. The growth performance, intestinal development, and transcription of AA transporters were determined. In experiment 2, 24 broiler chickens were subjected to the same treatments as in experiment 1. The plasma concentrations of free AAs were measured 0, 2, 4, and 6 h after feeding. The

results showed that 80CLM treatment had no significant influence on production performance, carcass characteristics, and plasma free AAs content during the experiment compared with the control group ($P > 0.05$). In addition, the 80CLM group moderately enhanced gut morphology development and increased AAs' absorption capacity. However, broilers fed the 60CLM diet had lower production performance and breast muscle weight than the control group ($P < 0.05$), but increased villi height and B⁰AT mRNA expression level ($P < 0.05$). At h 4 after feeding, the 60CLM broilers exhibited higher concentration of Ala, Cys, and total dispensable AAs than the control group ($P < 0.05$). In conclusion, the result suggests that the supplemental levels of crystalline Lys and DL-Met can be effectively saved approximately for 20% by using the encapsulated form in broilers, with improvements to AAs utilization efficiency, while posing no detrimental effects on production performance. Encapsulated Lys and DL-Met would have greater potential for application when replacing crystalline AAs in broiler chickens.

Key words: broiler, encapsulated amino acid, lysine, DL-methionine

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INTRODUCTION

Dietary supplementation with essential amino acids (AAs) increases the efficiency of utilization of dietary crude protein (Wang et al., 2009). Methionine (Met) and lysine (Lys) are considered as first and second rate-limiting AA in corn-soybean diets for poultry, respectively (Martívenegas et al., 2006; Nukreaw and Bunchasak, 2015). Lysine and DL-Met additions improve breast muscle yield and reduce abdominal fat

content in the carcass of male broiler chickens (Rakangtong and Bunchasak, 2011). Adversely, an imbalanced AAs composition results in an increased nitrogen loss in the urine, which increases the release of ammonia and in turn deteriorates the welfare status and impairs zootechnical performance (Beker et al., 2004; Patterson and Adrizal, 2005; Powers and Angel, 2008; Li et al., 2018).

In pigs, the crystalline AAs supplemented in the diet are absorbed quickly, compared with the protein-bound AAs, and appear in the portal blood vein more rapidly than protein-bound AAs; this may result in a transient imbalance among AAs (Sawadogo et al., 1997; Yen et al., 2004; Wu, 2009; Prandini et al., 2013). The rapid absorption of crystalline AAs may accelerate their oxidation and reduce their utilization efficiency (Boirie et al., 1997; Yen et al., 2004). The use of encapsulated AAs would resist gastric acidic condition,

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¹Corresponding authors: zjp1299@sdau.edu.cn (JZ); hailin@sdau.edu.cn (HL)

allowing a slow intestinal release (Prandini et al., 2013). Compared with both crystalline lysine-HCl and protein-bound Lys, the use of encapsulated Lys can save protein and synthetic AAs and reduce N excretion in manure without adversely affecting the growth performance and carcass quality of heavy growing-finishing pigs (Prandini et al., 2013). The supplementation of rumen protected Met increased plasma Met concentration, resulting in a small increase in milk protein percentage and to improve embryonic size and pregnancy maintenance in multiparous cows (Toledo et al., 2017). Supplementation of ruminally protected proteins and AAs enhanced digestion and improved the performance of cattle and sheep (Ali et al., 2009). Our previous work indicated that combination of encapsulated Lys and DL-Met could reduce the supplemental level of Lys and DL-Met in laying hens (M. Sun, unpublished data). Hence, we hypothesized that the encapsulated Lys and DL-Met would increase their AA utilization efficiency by ameliorating AA balance.

In the present study, 2 experiments were conducted to investigate the effect of encapsulated Lys and DL-Met on the production performance of broiler chickens. In experiment 1, the growth performance, plasma parameters, and carcass characteristics were examined. The mRNA levels of AA transporters, intestinal mucosa villus height, and crypt depth in the small intestinal segments were determined. In experiment 2, the plasma concentrations of free AAs in the blood at different post-prandial time points (0, 2, 4, and 6 h) were determined.

MATERIALS AND METHODS

All procedures in the study were approved by the Animal Care Committee of Shandong Agricultural University and were performed in accordance with the guidelines for experimental animals of the Ministry of Science and Technology (Beijing, China).

Crystalline Amino Acids

The crystalline DL-Met and Lys-HCl (99%) were obtained from a commercial company (Aladdin Industrial Corporation, Shanghai, China). The crystalline DL-Met and Lys-HCl were encapsulated within stearic acid matrix. The content of stearic acid was same for each dietary treatment. The DL-Met and Lys-HCl contents in the encapsulated products were 49.5%.

Experiment 1

A total of 432 male one-day-old Arbor Acres broiler chicks (44.13 ± 0.08 g) were obtained from a commercial farm (Dabao Breeding Technology Co., Taian, China). The broilers were randomly divided into 27 groups of sixteen birds and assigned to 3 dietary treatments (9 replicates per treatment). They were fed with a corn-soybean-based starter diet from 1 to 21 d of age and grower from 22 to 42 d of age supplemented with crystalline Lys-HCl and DL-Met (control), or the basal diet

supplemented, respectively, with encapsulated Lys-HCl and DL-Met at the levels of 60% and 80% of control (**60CLM** and **80CLM**), in accordance with our experiment on laying hens (M. Sun, unpublished data). The basal diet was formulated in accordance with the recommendations of the National Research Council (NRC, 1994). Ingredient and nutrient composition of the experimental diets are shown in Table 1. All the chicks were reared in an environmentally controlled chamber, and the rearing temperature was maintained at 33°C from day 1 to day 5, then gradually reduced to 22°C for the experimental period. Chickens were reared in battery cages (100 × 60 × 50 cm) with 375 cm² floor area per chicken. Each cage pen was equipped with feeders (the feeder space per bird was 6.2 cm) and nipple water line (4 birds per nipple). The light regimen was 23 L:1 D and the dark period was from 0:00 to 01:00 am (Zhao et al., 2012; Tang et al., 2019). During the rearing period, the broilers had free access to water and food.

Feed intake was recorded weekly and the BW was recorded at 21 and 42 d of age. At 21 and 42 d of age, one chicken around mean BW was randomly selected from each replicate. After overnight feed withdrawal, a blood sample was drawn from the wing vein using heparinized syringe from each chicken. The blood sample was collected with ice-cold tube. Plasma was obtained by centrifugation at 3,000 *g*, 4°C for 10 min and stored at -20°C for further analysis. Immediately after the blood sampling, broilers were sacrificed by exsanguination after cervical dislocation (Close et al., 1997; Huang et al., 2015). The duodenum, jejunum, and ileum were weighed and sampled at the midsection. Tissue samples were snap-frozen in liquid nitrogen and stored at -80°C before further analysis. Intestine segments (2.5 cm length) of the duodenum, jejunum, and ileum were fixed in 4% neutral buffered formalin for histological analysis. The liver, abdominal fat pad, and breast and thigh muscles were weighed.

Experiment 2

Twenty-four male 42-day-old Arbor Acres broiler chickens with similar BW (2.10 ± 0.02 kg) were randomly divided into 3 groups of eight broilers. After 12-h feed withdrawal, a blood sample was drawn from the wing vein (0 h) using heparinized syringe from each chicken. Then, the broilers were fed 40 g diets of control, 80CLM, and 60CLM grower diets aforementioned in experiment 1, respectively. All the feed was consumed within 30 min. Blood samples were collected via wing vein at 2, 4, and 6 h after feeding, respectively. Plasma samples were obtained after centrifugation at 3,000 *g* for 15 min at 4 °C and stored at -20 °C for further analysis.

Dietary Crude Protein and Amino Acid Analysis

The experimental diets were analyzed for dry matter (method 930.15), crude protein (method 990.03),

Table 1. Ingredient and nutrition composition of experiment diets.

Item	Starter (day 1-21)			Grower (day 22-42)		
	Control	80CLM ¹	60CLM ¹	Control	80CLM ¹	60CLM ¹
Ingredients (%)						
Corn	60.47	60.22	60.39	64.07	63.87	64.00
Soybean meal (46%)	33.74	33.74	33.74	28.84	28.84	28.84
Soybean oil	1.50	1.50	1.50	3.00	3.00	3.00
Limestone	1.04	1.04	1.04	1.17	1.17	1.17
Dicalcium phosphate	2.00	2.00	2.00	1.85	1.85	1.85
Salt	0.32	0.32	0.32	0.29	0.29	0.29
Lysine-HCl (99%)	0.17	-	-	0.15	-	-
DL-methionine (99%)	0.25	-	-	0.18	-	-
Choline chloride (50%)	0.26	0.26	0.26	0.20	0.20	0.20
Encapsulated lysine-HCl	-	0.272	0.204	-	0.24	0.18
Encapsulated methionine	-	0.40	0.30	-	0.288	0.216
Vitamin premix ²	0.05	0.05	0.05	0.05	0.05	0.05
Microelements ³	0.20	0.20	0.20	0.20	0.20	0.20
Nutrition level ⁴ , kcal/kg, %						
ME	2,900	2,900	2,900	3,030	3,030	3,030
CP	20.19	20.07	20.19	18.61	18.60	18.49
Ca	0.98	1.06	1.02	1.06	1.05	1.00
AP	0.48	0.47	0.46	0.43	0.44	0.42
Lys	1.225	1.172	1.111	1.057	1.024	1.002
Met	0.513	0.472	0.415	0.465	0.423	0.366
Met + Cys	0.882	0.821	0.786	0.797	0.765	0.724
Thr	0.829	0.822	0.823	0.719	0.715	0.716
Val	1.051	1.042	1.045	0.848	0.844	0.835
Ile	0.903	0.893	0.898	0.697	0.695	0.686
Leu	1.525	1.533	1.523	1.497	1.498	1.485
Phe	1.229	1.220	1.225	1.067	1.056	1.072
Arg	1.011	0.982	0.995	0.779	0.768	0.775
Gly	1.055	1.074	1.061	0.740	0.740	0.747
Asp	2.778	2.777	2.768	1.855	1.849	1.852
Ser	1.065	1.072	1.078	0.856	0.852	0.843
Glu	4.079	4.094	4.081	3.029	3.020	3.024
Ala	1.250	1.228	1.241	0.917	0.922	0.921
His	0.994	1.024	1.015	0.638	0.646	0.642

Abbreviations: Ca, calcium; AP, phosphorus.

¹60CLM and 80CLM: broilers fed with the basal diet supplemented with encapsulated Lys and DL-Met at the levels of 60% and 80% of the control diet, respectively.

²Premix provided per kg compound feed: vitamin A, 44 IU; vitamin D, 12 IU; vitamin E, 140 IU; vitamin K₃, 6.3 g; vitamin B₁, 6.1 g; vitamin B₂, 18.8 g; vitamin B₆, 9.2 g; vitamin B₁₂, 5.0 g; niacin, 81.2 g; pantothenic acid, 24.4 g; folic acid, 2.0 g; wheat-middlings, 651.1 g.

³Premix provided per kg compound feed: CuSO₄·5H₂O, 15.7 g; FeSO₄·H₂O, 15.7 g; MnSO₄·5H₂O, 193.5 g; ZnSO₄·H₂O, 166.7 g; Ca(IO₃)₂, 0.9 g; Na₂SeO₃, 0.3 g; and limestone powder, 461.6 g.

⁴All the listed nutrient levels were measured value except of the ME (kcal/kg). The unit of other nutrient levels was %.

calcium (method 984.01), and phosphorus (method 965.17) of basal diet as described by [AOAC International \(1996\)](#). Dietary AAs were determined by ion-exchange chromatography using a Hitachi L-8900 AA Analyzer (Tokyo, Japan) after acid hydrolysis with 6 N HCl and reflux for 24h. Methionine and cysteine were analyzed as Met sulfone and cysteic acid after cold performic acid oxidation overnight before hydrolysis.

Plasma Parameters and Free Amino Acids

In experiment 1, the alanine aminotransferase (ALT), aspartate aminotransferase (AST), urate, and urea nitrogen (urea-N) were measured by the Hitachi L-7020 fully automatic biochemical analyzer (Tokyo, Japan). A commercial assay kit (AA-1-W, Keming, Suzhou, China) was used to determine the content of total free amino acids (TFAA) in plasma, in accordance with the manufacturer's instructions. In experiment 2, plasma free AA concentrations were determined by ion-

exchange chromatography using a Hitachi L-8900 AA Analyzer (Tokyo, Japan) under physiological fluid analysis conditions. The plasma sample (800 µL) were thawed at 4°C, deproteinized with 40 mg of salicylic acid, and then blended with an oscillator (Guohua Electric Appliance, China). After stewing for 1 h at 4°C, the samples were centrifuged at 12000g for 30 min. The supernatant fluid was collected and passed through a filter (0.1 µm) before AA analysis.

Intestinal Morphology

Formalin-fixed duodenum, jejunum, and ileum samples were embedded in paraffin and cut into 5-µm serial sections. Three sections from each tissue sample were selected and stained with hematoxylin-eosin for identification. Six well-oriented villi and their associated crypt were selected for each section, measured under a light microscope (CK-40, Olympus, Tokyo, Japan) at 40 × magnification and analyzed with an Image

Table 2. Primers used for real-time PCR.

Gene	Genebank accession no.	Orientation	Sequences (5'-3')	Product size (bp)
CAT1	NM_001145490.1	Forward	GCTCTATGGTGTGGAGGG	192
		Reverse	AATAAGCCACAAAAGCAGATGAG	
b ⁰⁺ AT	NM_001199133.1	Forward	TGTGTTGCTCTCTAACTGGCTG	154
		Reverse	CCTCCTTTCTGTTGTCCTGTTC	
y ⁺ LAT1	XM_418326.5	Forward	CATTCTCAGGGTTTCAGAGCAC	216
		Reverse	CTGTCCTTTCTCCCATCGTG	
rBAT	XM_004935370.2	Forward	TTGGCTTGGCAAAGGAGTC	146
		Reverse	TCGGAATAGGCTGTGATGCT	
B ⁰ AT	XM_419056	Forward	AATGGGACAACAAGGCTCAG	125
		Reverse	CAAGATGAAGCAGGGGGATA	
EAAT3	XM_424930	Forward	ACCCTTTTGCCTTGGAAACT	122
		Reverse	TTGAGATGTTTGCCTGAAG	
PepT1	NM_204365	Forward	ACACGTTTGTGCTCTGTGC	122
		Reverse	GACTGCCTGCCAATTGTAT	
β-Actin	XM_003357928	Forward	TGCGGGACATCAAGGAGAAG	216
		Reverse	AGTTGAAGGTGGTCTCGTGG	

Abbreviations: CAT-1, cationic amino acid transporter-1; b⁰⁺AT, b⁰⁺ amino acid transporter; y⁺LAT1, y⁺L amino acid transporter-1; rBAT, related to b⁰⁺ amino acid transporter; B⁰AT, B⁰ neutral amino acid transporter; EAAT3, acidic amino acids transporter; PepT1, intestinal peptide transporter-1. Tm, melting temperature.

Analyzer (Lucia Software, Lucia, Za Drahou, Czechia). The 18 measurements were averaged to yield 1 value per chicken. These procedures were conducted by an observer unaware of the dietary treatments to avoid bias.

RNA Isolation and RT-PCR Analysis

Total RNA was isolated from each intestinal segment (~100 mg, frozen in liquid nitrogen) with the TRIZOL reagents (Invitrogen, CA). RNA quality was determined with agarose gel electrophoresis and a spectrophotometry (Eppendorf, Germany) detecting the UV absorbance ratio at 260 nm and 280 nm (A₂₆₀/A₂₈₀ ≈ 1.75-2.01). Then 1 µg RNA was reverse-transcribed to complementary DNA using DNase I (Invitrogen) in accordance with the manufacturer's protocol. Primers were designed with the use of DNAMAN software on the base of gene sequences of chicken (Table 2). Beta-actin was used as an internal reference gene to normalize target gene transcript level. Real-time PCR was

performed using an Applied Biosystems Quantstudio 5 Real-time PCR system (Foster City, CA). An RNA standard curve for each gene was generated based on modification of the protocol. The system included, 2 µL DNA template, 10 µL SYBR Green mix (containing MgCl₂, dNTP, and Hotstar Taq polymerase), 7 µL deionized H₂O and 0.5 µL each of forward and reverse primers in a total volume of 20 µL. The protocols included predenaturation (10 s at 95°C), amplification, and quantification program repeated at 40 cycles (5 s at 95°C, 34 s at 60°C) and melting curve program extension at 75°C. Eight samples were used per treatment and each sample was measured in triplicate. The relative expression of genes was compared with the control group using cycle threshold (Ct) values (Fu et al., 2006).

Statistical Analysis

Before analysis, all data were examined for the homogeneity and normal distribution plots of variances

Table 3. Effect of encapsulated Lys and DL-Met supplementation on the production performance of broiler chickens.¹

Item	Control	80CLM ²	60CLM ²	P-value
Day 1-21				
BW gain, g/d	33.59 ± 0.58 ^a	32.04 ± 0.58 ^{ab}	30.61 ± 0.45 ^b	0.005
ADFI, g/d	46.24 ± 0.52	47.05 ± 0.85	46.57 ± 0.95	0.775
F:G, g/g	1.39 ± 0.02 ^a	1.47 ± 0.02 ^{ab}	1.53 ± 0.05 ^b	0.010
Day 22-42				
BW gain, g/d	67.43 ± 3.24	64.47 ± 3.33	65.19 ± 2.74	0.785
ADFI, g/d	131.46 ± 3.45	124.23 ± 4.59	124.53 ± 3.23	0.331
F:G, g/g	1.97 ± 0.06	1.94 ± 0.04	1.92 ± 0.04	0.771
Day 1-42				
BW gain, g/d	50.93 ± 1.68	48.58 ± 1.87	48.39 ± 1.43	0.497
ADFI, g/d	87.67 ± 1.79	83.96 ± 2.42	83.84 ± 1.72	0.335
F:G, g/g	1.73 ± 0.03	1.73 ± 0.03	1.74 ± 0.02	0.973
Mortality, %	3.71 ± 1.17	3.71 ± 1.17	4.46 ± 1.57	0.901

^{a,b}Means with different superscripts within the same column differ significantly, $P < 0.05$.

¹Data were presented as mean ± SEM (n = 9).

²60CLM and 80CLM: broilers fed with the basal diet supplemented with encapsulated Lys and DL-Met at the levels of 60% and 80% of control diet, respectively.

among the treatments by using UNIVARIATE procedure. In experiment 1, for variables feed intake, BW gain, FCR, organ index, plasma variables, and gene expressions, a one-way ANOVA model was used to estimate the main effects of dietary treatment (SAS version 8.1; SAS Institute Inc., NC). When the main effect of the treatment was significant, the differences between means were compared using Tukey's multiple comparisons test. To analyze for the plasma free AAs in experiment 2, the repeated measurement analysis was conducted to estimate the main effects of diet with each chicken as replicate. Paired T-test was used to evaluate the time effect within treatment. $P < 0.05$ was considered statistically significant.

RESULTS

Experiment 1

During the starter phase, the BW gain was decreased while the feed-to-gain ratio was elevated in 60CLM broilers compared with control ($P < 0.05$, Table 3). By contrast, neither BW gain nor feed-to-gain ratio was significantly changed ($P > 0.05$) by dietary treatment at grower stage and throughout the experimental period. Feed intake was not influenced ($P > 0.05$) by dietary treatment at the starter, grower, and the entire whole experimental period. There was no detectable difference in mortality between treatments ($P > 0.05$).

At day 42, the breast muscle and liver weights were decreased ($P < 0.05$) significantly in the 60CLM diet compared with the control (Figures 1A, 1B). However, there were no obvious differences in the thigh muscle, abdominal fat pad, duodenum, jejunum, and ileum among the 3 dietary treatments ($P > 0.05$).

At day 21, 60CLM broilers had higher ($P < 0.05$) plasma AST activity and TFAA concentration compared with the 80CLM and control group, respectively (Table 4). The ALT, urate, and urea-N levels were not changed by dietary treatments ($P > 0.05$).

At day 42, no significantly difference ($P > 0.05$) was observed in the plasma ALT, AST, urate, urea-N, and TFAA among the 3 dietary treatments (Table 4).

The villi height was not influenced ($P > 0.05$) by dietary treatment in the duodenum and ileum, except the jejunum, where the 60CLM broilers had higher villi height than the control ($P < 0.05$, Figure 2A). The crypt depth was not changed by dietary treatment ($P > 0.05$, Figure 2B). By contrast, the ratio of villi height to crypt depth in the duodenum was significantly changed by dietary treatment because the 80CLM broilers had a higher ratio (+29.9%) compared with the control ones ($P < 0.05$, Figure 2C). The representative diagrams on morphology of the duodenal, jejunal, and ileal mucosae are shown in Figure 2D.

The mRNA expression levels of AA transporters *rBAT*, *b^{0,+}AT*, *EAAT3*, *CAT1*, and *y⁺LAT1* in the duodenum, jejunum, and ileum were not altered by dietary treatment ($P > 0.05$, Figures 3A, 3B, 3E, 3F, 3G). The *PepT1* mRNA level in the ileum, however, was decreased in 80CLM broilers compared with control ($P < 0.05$, Figure 3C). The *B⁰AT* mRNA level was upregulated ($P < 0.05$) by 60CLM treatment in the duodenum and ileum, compared with the control and 80CLM group, respectively (Figure 3D).

Experiment 2

The AAs content in plasma were measured at 0, 2, 4, and 6 h time points after feeding to evaluate the effect of dietary AA treatment on AA metabolism. Dietary treatment had no significant influence on the concentration of Lys, Met, Gly, Tau, total indispensable AA (TIAA) and total AA (TAA) at all the measurement time points ($P > 0.05$, Figures 4A, 4B, 4C, 4F, 4G and 4I). However, at the 4-h time point, compared with the control group, 60CLM treatment exhibited higher levels of Ala, Cys, and total dispensable AA (TDAA) ($P < 0.05$, Figures 4D, 4E, 4H), whereas there were no obvious differences ($P > 0.05$) at other time points among these

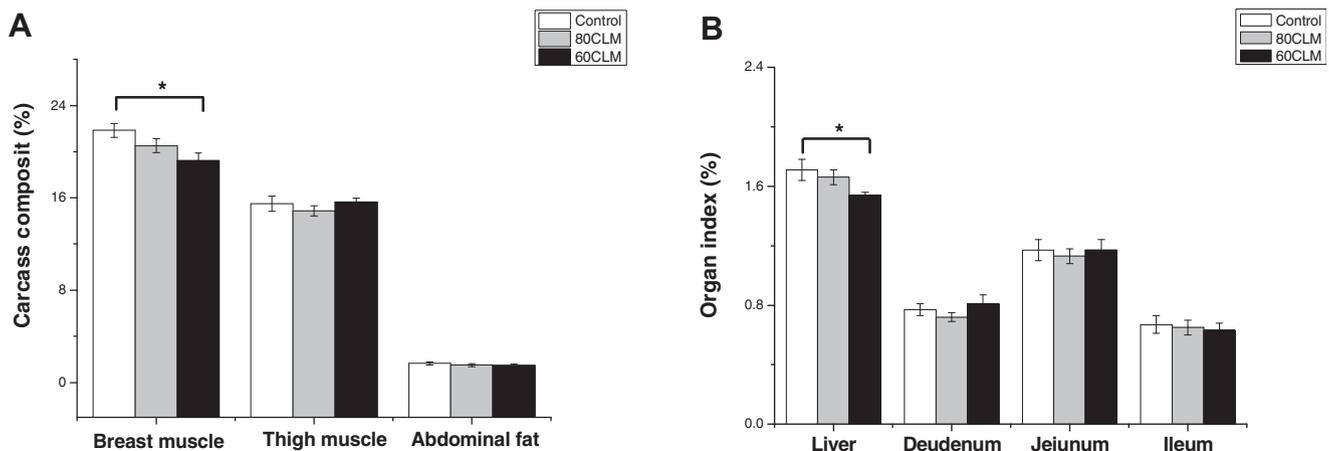


Figure 1. Effect of encapsulated Lys and DL-Met supplementation on carcass composition (A) and relative weight of organs (B) of birds at 42 d of age. Data were presented as mean \pm SD (n = 9); 60CLM and 80CLM: broilers fed with the basal diet supplemented with encapsulated Lys and DL-Met at the levels of 60% and 80% of the control diet, respectively. * $P < 0.05$.

Table 4. Effect of encapsulated Lys and DL-Met supplementation on plasma concentrations of ALT, AST, urate, urea-N, and TFAA at the 21 and 42 d of age.¹

Item	Control	80CLM ²	60CLM ²	P-value
Day 21				
ALT, U/L	7.35 ± 0.16	7.72 ± 0.31	7.52 ± 0.18	0.699
AST, U/L	209.88 ± 5.64 ^{a,b}	188.75 ± 11.49 ^b	228.75 ± 12.70 ^a	0.042
Urate, µmol/L	266.63 ± 59.05	253.75 ± 43.41	273.63 ± 53.02	0.964
Urea-N, mmol/L	0.69 ± 0.04	0.66 ± 0.04	0.72 ± 0.03	0.510
TFAA, µmol/mL	1.77 ± 0.29 ^b	2.14 ± 0.32 ^{a,b}	3.18 ± 0.49 ^a	0.041
Day 42				
ALT, U/L	7.90 ± 0.32	8.15 ± 0.26	7.70 ± 0.15	0.597
AST, U/L	255.43 ± 17.93	260.50 ± 13.02	270.88 ± 25.40	0.854
Urate, µmol/L	288.57 ± 32.36	205.38 ± 30.38	257.50 ± 27.52	0.170
Urea-N, mmol/L	0.74 ± 0.05	0.72 ± 0.04	0.72 ± 0.07	0.964
TFAA, µmol/mL	7.40 ± 1.22	6.44 ± 0.75	5.16 ± 0.50	0.213

^{a,b}Means with different superscripts within the same column differ significantly, $P < 0.05$.

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; Urea-N, urea nitrogen; TFAA, total free amino acids.

¹Data were presented as mean ± SD (n = 9).

²60CLM and 80CLM: broilers fed with the basal diet supplemented with encapsulated Lys and DL-Met at the levels of 60% and 80% of control diet, respectively.

dietary treatments. The results of other AAs content in plasma at 0, 2, 4, and 6 h time points after feeding are shown in [Supplementary Table 1](#).

The Gly, Ala, Tau, Cys, TDAA, and TAA showed different trends with postprandial time for the 3 dietary treatments. At the 2 h time point after feeding, plasma Tau was increased in the control birds ($P < 0.05$), and 80CLM birds improved the Gly and Ala levels, compared with the 0-h time point ($P < 0.05$). In the control birds, plasma Cys and Tau were reduced at the 4-h time point compared with the 2 h time point ($P < 0.05$), as well as Gly, Ala, TDAA, and TAA ($P < 0.01$). At the 6 h time point, the plasma Gly, Ala, and Tau remained lower levels ($P < 0.05$), compared with the 2 h time point. In the 80CLM birds, Gly and TAA ($P < 0.01$) and Ala and TDAA ($P < 0.05$) were declined by h 4, whereas Gly ($P < 0.01$), Ala ($P < 0.01$), and TAA ($P < 0.05$) were reduced by the 6 h time point compared with the 2 h time point. In the 60CLM birds, however, only Gly and TAA had decreased by 4 h ($P < 0.05$), but there were no differences among these AAs by the 6-h time point ($P > 0.05$). The Lys, Met, and TIAA levels had no obvious difference with time in the different dietary treatments ($P > 0.05$).

DISCUSSION

The results indicated that supplementation with encapsulated Lys and DL-Met at 80% of control levels had no negative effect on the production performance of broilers. The finding suggests that encapsulated AA could improve the utilization efficiency of crystalline AA by ameliorating AA balance after absorption in broilers.

Effects of Encapsulated Lys and DL-Met on the Growth Performance of Broilers

Crystalline AAs have high nutritional values when they are added to a low protein diet or a diet deficient in those AAs ([Baker, 2009](#)). As the first and second

rate-limiting AA in corn-soybean based diet for poultry, crystalline Met and Lys have been extensive used as feed additive in poultry production. Increasing dietary Met and Lys levels significantly improves breast muscle yield and reduced abdominal fat content in the carcass of male broiler chickens ([Rakangtong and Bunchasak, 2011](#)). In the present study, the BW gain, ADFI, feed-to-gain ratio, and carcass composition were not significantly changed by 80CLM treatment, suggesting the supplemental levels of dietary Lys and Met were adequate. This was supported by the observation that there were no significant differences in plasma urate, urea-N, and TAA levels between control and 80CLM treatments. Urate is the major end product of nitrogen metabolism in birds and can be used as an indicator of AA utilization in broilers fed AA-adequate and AA-deficient diets ([Whang et al., 2003](#); [Namroud et al., 2008](#); [Donsbough et al., 2010](#)). Therefore, the results indicated that 80CLM increases AA utilization efficiency compared with control treatment by using encapsulated AA. By contrast, the BW gain and feed efficiency during the starter stage and the relative breast muscle weight at 42 d of age were reduced in broilers fed the 60CLM diet. This suggests that the 60CLM Lys and Met supply could not satisfy the AA requirement during starter period and for initiation of breast muscle growth throughout the rearing stage. This finding was supported by the observation that 60CLM chickens had higher plasma TAA concentration at 21 d of age, compared with the control. Collectively, the result suggests that supplementation of Lys and DL-Met in an encapsulated form can salvage the supplemental levels of these AAs without detrimental effects to the growth performance of broilers.

Effects of Encapsulated Lys and DL-Met on AA Transportation

Dietary AAs are essential in maintaining the small intestinal integrity as well as function, by serving as the

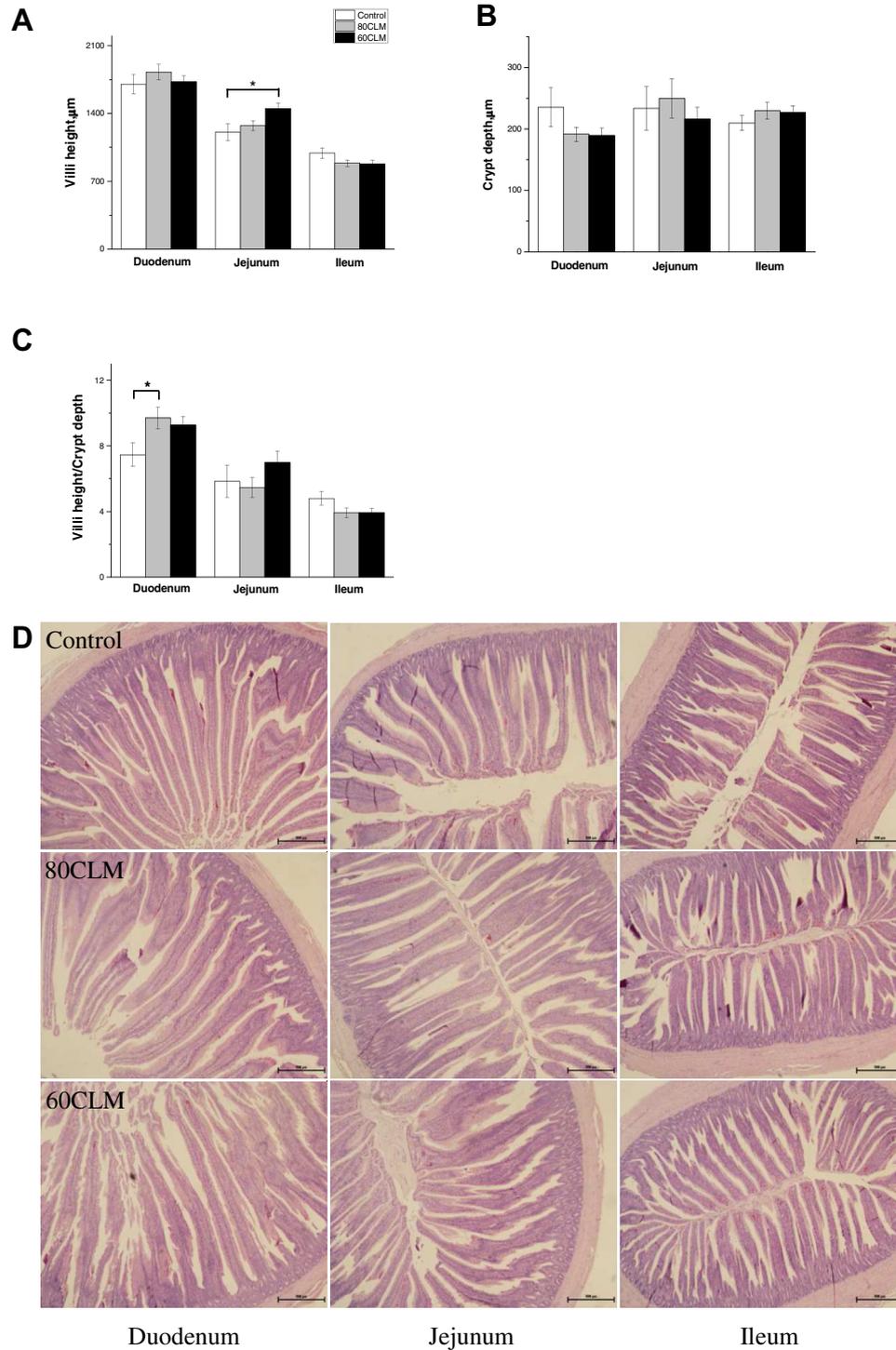


Figure 2. The morphology of intestine of broiler chicks at 21 d of age. (A) Villi height; (B) crypt depth; (C) villi height to crypt depth ratio; (D) the morphology of the duodenum, jejunum, and ileum. Data were presented as mean \pm SD ($n = 6$); 60CLM and 80CLM: broilers fed with the basal diet supplemented with encapsulated Lys and DL-Met at the levels of 60% and 80% of the control diet, respectively. * $P < 0.05$.

primary fuels for energy and precursor of protein synthesis in the intestinal mucosa (Bergen and Wu, 2009; Wang et al., 2009). Villi height and crypt depth are the important exchange areas for digestion and absorption. A normal structure of the small intestinal mucosa is necessary for optimal growth as well as nutrient digestion and absorption (Thompson and Applegate, 2006; Song et al., 2014; Wang et al., 2015; Bai et al., 2018).

In the present study, the results showed that dietary supplementation with encapsulated Lys and DL-Met at 60% or 80% of control level had no detrimental effects on the histomorphology of the intestinal tract. The increased villi height in the jejunum of the 60CLM group may be a compensatory effect of the reduced dietary Lys and DL-Met. Villi height and surface area make up the area available for digestion/absorption, and the

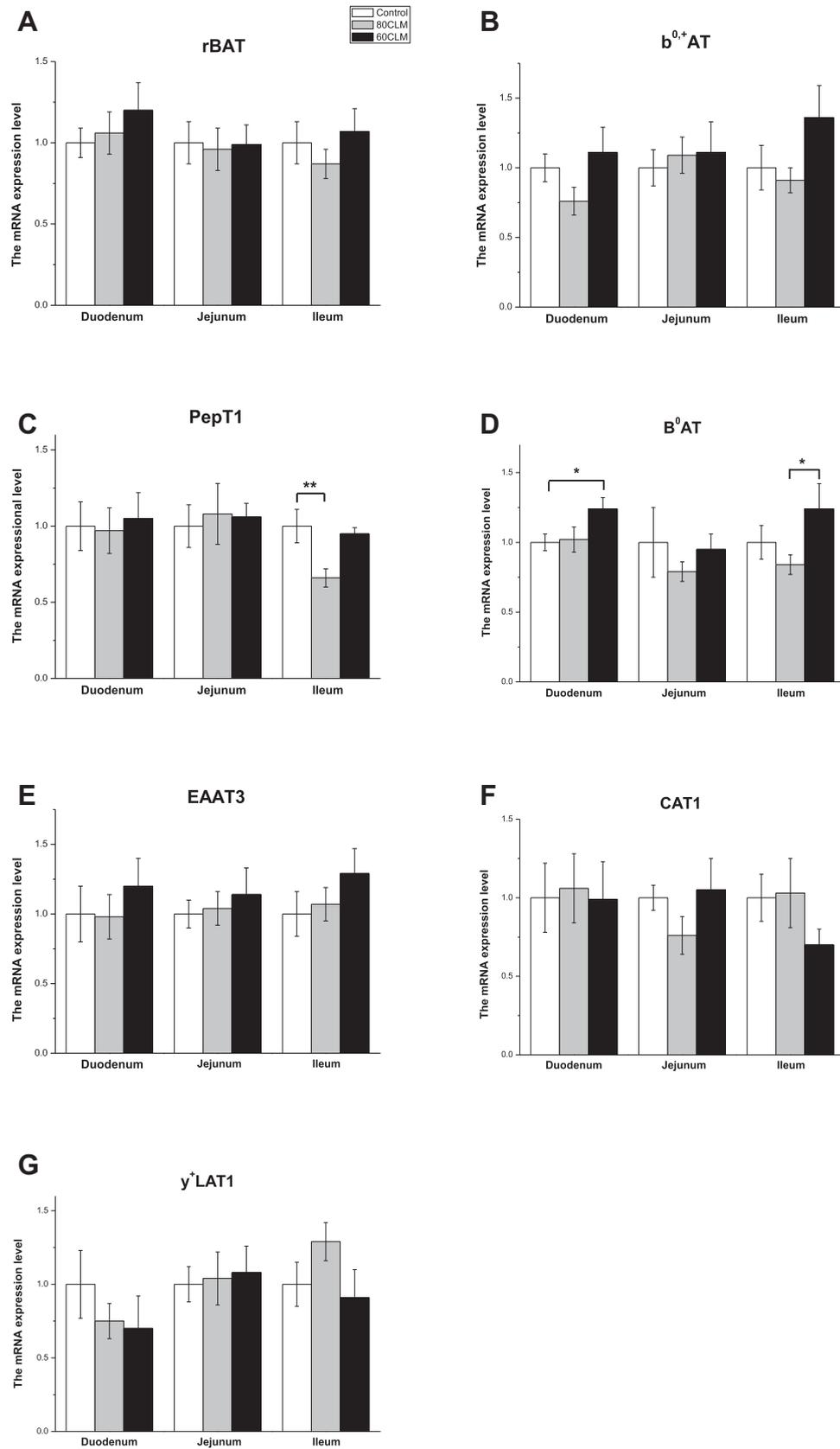


Figure 3. Effect of encapsulated Lys and DL-Met supplementation on the gene expression of *rBAT* (A), *b^{0,+}AT* (B), *PepT1* (C), *B⁰AT* (D), *EAAT3* (E), *CAT1* (F), and *γ^+LAT1* (G) in different sections of intestinal tract in broiler chicks at 21 d of age. Data were presented as mean \pm SD (n = 8); 60CLM and 80CLM: broilers fed with the basal diet supplemented with encapsulated Lys and DL-Met at the levels of 60% and 80% of the control diet, respectively. * $P < 0.05$, ** $P < 0.01$.

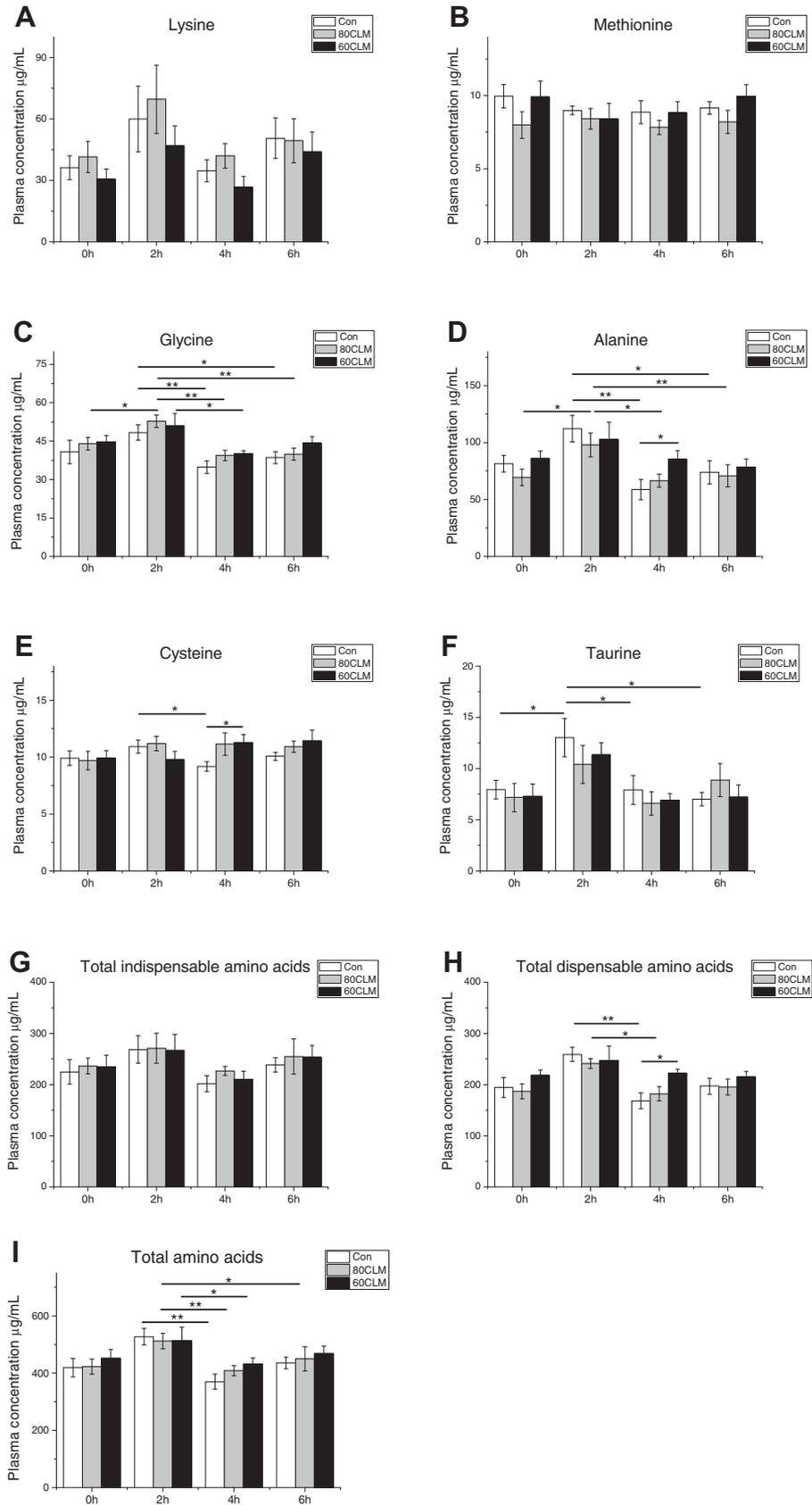


Figure 4. Effect of encapsulated Lys and DL-Met supplementation on postprandial plasma lysine (A), methionine (B), glycine (C), alanine (D), cystine (E), taurine (F) and total indispensable amino acids (G), total dispensable amino acids (H), and total amino acids (I) concentration ($\mu\text{g}/\text{mL}$) at 0, 2, 4, and 6 h time points. Data are shown as mean \pm SD ($n = 8$). 60CLM and 80CLM: broilers fed with the basal diet supplemented with encapsulated Lys and DL-Met at the levels of 60% and 80% of the control diet, respectively. * $P < 0.05$, ** $P < 0.01$.

activities of membrane bound digestive enzymes of the small intestine (Iji et al., 2001). The villi height of chicks under *ad libitum* feeding regime, were higher for the chicks fed balanced AA mixture compared with those fed an imbalanced AA diet (Swatson et al., 2002).

The content of AAs in a diet can affect AA transporters expression (Gilbert et al., 2008a,b; He et al., 2013; Morales et al., 2019). AAs are transported into the enterocyte as free AAs by a variety of transporters that vary in substrate specificity or as dipeptides and tripeptides by the peptide transporter (Broer, 2008; Gilbert et al., 2008a,b; Zhang et al., 2013; Qiu et al., 2016). They constitute the basic AA transporters (CAT1, y⁺LAT1), neutral AA transporters (B⁰AT1, b^{0,+}AT), and acidic AA transporters (EAAT1, EAAT3) (Kanai and Hediger, 2003). Dietary protein quality and AA balance influences the abundance of nutrient transporter mRNA in small intestinal of broiler chicks (Gilbert et al., 2008a,b). The gene expression of *CAT1*, *y⁺LAT1*, and *b^{0,+}AT* were upregulated in the small intestine by increasing dietary lysine levels (He et al., 2013). In the present study, although the 60CLM diet had higher mRNA expression of *B⁰AT* in the duodenum and ileum, the relative abundances of intestinal AA transporters *CAT1*, *y⁺LAT1*, *b^{0,+}AT*, *rBAT*, and *EAAT3* were not changed by dietary treatments. Collectively, the result suggests that AA transportation was not obviously altered by encapsulated Lys and DL-Met.

Effects of Encapsulated Lys and DL-Met on AA Metabolism

The postprandial plasma AA profile reflects the AA composition of the diet through the rates of digestion, absorption, and first-pass metabolism in splanchnic tissues (Gloaguen et al., 2012). In the present study, the AA concentrations were measured in the blood sampled from wing vein as described by previous researchers (Bos et al., 2003; Yen et al., 2004; Pennings et al., 2011; Prandini et al., 2013). Hence, the present result actually reflected a result of digestion, absorption, and whole-body metabolism. The insignificantly change of plasma Lys and Met concentrations by dietary treatment indicated that reducing the level of Lys and DL-Met via the supplementation of their encapsulated form had no alterations on their metabolism. The different changing trends for plasma Gly, Ala, Cys, Tau, and TDAA concentrations with postprandial time in the control, 80CLM, and 60CLM broilers, however, implied that the encapsulated Lys and DL-Met treatment influenced the metabolism of other AAs, in line with our previous result in laying hens (M. Sun, unpublished data). Crystallite AAs, compared with protein-bound AAs, are known to be sensitive to acidic conditions and rapidly absorbed in the digestive tract (Yen et al., 2004; Morales et al., 2019). The encapsulated Lys and DL-Met can protect AA by resisting gastric acidic condition and allowing an intestinal slow release (Piva et al., 2007). In a pig model, the crystalline lysine and

threonine were absorbed more rapidly than protein-bound lysine and threonine in pigs fed once daily (Yen et al., 2004). Hence, the present result suggests that encapsulated Lys and Met can modify AA metabolism in broilers.

In conclusion, the result suggests that the supplemental levels of synthetic Lys and DL-Met can be effectively decreased approximately by 20% via the encapsulated form without significant alterations and adverse effects on the production performance of broilers. To our knowledge, this is the first study to report that encapsulated forms of AA have the capacity to improve AA utilization efficiency in broilers.

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Conflict of Interest Statement: The authors declare no conflicts of interest.

SUPPLEMENTARY DATA

Supplementary data associated with this article can be found in the online version at <https://doi.org/10.1016/j.psj.2020.09.023>.

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