NON RUMINANT NUTRITION

Use of encapsulated L-lysine-HCl and DL-methionine improves postprandial amino acid balance in laying hens

Mingfa Sun, Jingpeng Zhao, Xiaojuan Wang, Hongchao Jiao,¹ and Hai Lin¹

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

¹Corresponding author: hongchao@sdau.edu.cn; hailin@sdau.edu.cn

Abstract

The supplementation of dietary limiting amino acids (AA) with crystalline AA makes the use of low-protein diets an option in poultry production. The differing absorption rates of crystalline and protein-bound AA may lead to temporally imbalanced AA in the postabsorptive period. In this study, two experiments were conducted to evaluate the effect of encapsulated L-lysine-HCl (**L-Lys-HCl**) and DL-methionine (**DL-Met**) on the laying performance of hens. In exp. 1, a total of 135 forty-seven-wk-old Hy-Line Brown hens were subjected to three dietary treatments for 8 wk: basal diet supplemented with 0.14% L-Lys-HCl and 0.17% DL-Met to satisfy the [NRC \(1994\)](#) total Lys and Met recommendation (control) and basal diet supplemented with encapsulated L-Lys-HCl and DL-Met at the levels of 60% (**60CLM**, 0.084% L-Lys-HCl and 0.102% DL-Met) or 80% of control (**80CLM**, 0.112% L-Lys-HCl and 0.136% DL-Met), respectively. In exp. 2, 24 fifty-five-wk-old Hy-Line Brown hens were individually reared in cages and subjected to the same treatments as in exp. 1. The plasma concentrations of free AA and nitrogen metabolites were measured 2, 4, and 6 h after fed. The results showed that dietary AA treatment had no significant influence on body weight (BW), feed intake, laying rate, egg weight, egg mass, or feed efficiency. The expression levels of AA transporters CAT-1, γ -LAT1, b^0 -AT, B^0 AT, rBAT, EAAT3, and *Pept1* in the duodenum, jejunum, and ileum were not influenced ($P > 0.05$) by dietary treatment. There was an interaction of dietary AA treatment and time ($P < 0.05$) and the 80CLM hens exhibited higher concentrations of Lys ($P < 0.05$) than the controls at 2-h time point. In contrast, plasma Met concentration was not influenced ($P > 0.05$), while Cys was reduced in the 60CLM hens at every time point. The 80CLM hens had higher taurine concentrations than those receiving the control diet at every postprandial time point. In conclusion, these findings demonstrate that by using encapsulated form, the supplemental levels of synthetic L-Lys-HCl and DL-Met can be effectively reduced by approximately 20% with no negative effect on laying performance. The result suggests that encapsulated Lys and Met may ameliorate the postabsorptive AA balance and contribute to the reduced dietary AA supplemental levels.

Key words: dl-methionine, encapsulated amino acid, l-lysine-HCl, laying hen

Introduction

Dietary supplementation with certain amino acids (AA) can increase the efficiency of protein anabolism and reduce

feeding cost in animal husbandry ([Tan et al., 2009](#); [Wang et al., 2009](#)). AA not only serve as building blocks for protein but also are regulators of gene expression as cell signaling molecules

Abbreviations

AA	amino acids
ALT	alanine aminotransferase
AP	phosphorus
AST	aspartate aminotransferase
BW	body weight
CP	crude protein
DL-Met	DL-methionine
DM	dry matter
DNA	deoxyribose nucleic acid
L-Lys-HCl	L-Lysine-HCl
L-PA	L-pipecolic acid
ME	metabolizable energy
PCR	polymerase chain reaction
RNA	ribonucleic acid
TAA	total free amino acids
TDAA	total dispensable amino acids
TIAA	total indispensable amino acids
Urea-N	urea nitrogen

and in the protein phosphorylation cascade, also give rise to various metabolites (Wu, 2009; Yao et al., 2012). Methionine (Met) and lysine (Lys) are the first- and second-limiting AA in corn-soybean meal-based diets for poultry (Novak et al., 2004). Feeding laying hens diets that are deficient in Met and Lys results in suppressed whole-body protein synthesis and, more severely, reduction in the protein synthesis in the liver, magnum, and the remainder of the oviduct (Hiramoto et al., 1990). Moreover, Met is the precursor of some important active substances, such as glutathione, taurine, and carnitine (Vazquez-Anon et al., 2006; Wang et al., 2009). On the other hand, excessive supplementation with Met and Lys may negatively affect metabolism and performance (Han and Baker, 1993; Ghoreyshi et al., 2019). For example, L-pipecolic acid (L-PA), a major metabolic intermediate of Lys, has a detrimental influence on the feeding behaviors of chicks (Takagi et al., 2001).

Supplementation with crystalline AA of a diet deficient in AA balances the AA and improves the protein biological value (Mateo et al., 2008; Baker, 2009; Elango et al., 2009; Pennings et al., 2011). Dietary supplementary crystalline AA are readily absorbed, while protein-bound AA need to be hydrolyzed to small peptides and AA that can be absorbed by the small intestine (Rérat et al., 1992; Morales et al., 2015). Crystalline Lys and Thr were absorbed more rapidly than the same AA bound to proteins in pigs fed once daily (Yen et al., 2004). Rapid absorption of crystalline AA may lead to a transient imbalance among AA in the systemic circulation (Wu, 2009). The imbalanced AA can result in at least two outcomes: metabolic disorder, such as the suppression of food intake (Gloaguen et al., 2012), and excess AA catabolism and oxidation (Boirie et al., 1997; Yen et al., 2004; Li et al., 2018).

The use of microencapsulation of active ingredients in a matrix could enable slow intestinal release (Piva et al., 2007). Supplying rumen-protected DL-Methionine (DL-Met) to cows has been shown to protect Met from ruminal degradation and made it available for subsequent absorption in the small intestine (Wright and Loerch, 1988; Chow et al., 1990; Overton et al., 1998; Yang et al., 2010). Compared with both crystalline Lys and protein-bound Lys, the use of microencapsulated Lys has been shown to save protein and synthetic AA in diet formulation and reduce nitrogen excretion in manure, without adversely affecting the growth performance or carcass quality

of heavy growing-finishing pigs (Prandini et al., 2013). Hence, we hypothesized that encapsulated AA may cut down the supplemental level of crystalline AA by ameliorating the absorption rates of crystalline AA.

The objective of the present study was to investigate that if the encapsulated form could save the use of crystalline AA in laying hens. Two experiments were conducted to evaluate the effect of encapsulated L-lysine-HCl (L-Lys-HCl) and DL-Met in laying hens. In exp. 1, the laying performance, egg quality, plasma parameters, the morphology of small intestinal tract, and mRNA levels of AA transporters in the small intestine were determined to determine the effect of encapsulated AA treatment on laying performance and AA transportation. In exp. 2, the postprandial amino acid profiles were measured at 2, 4, and 6 h time points after fed to evaluate the effect of dietary AA treatment on AA metabolism.

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 AA

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

Experiment 1

One hundred thirty-five 47-wk-old Hy-Line Brown laying hens, of similar body weight (BW, 2.09 ± 0.06 kg), were used. The hens were randomly divided into 15 groups of nine hens and assigned to three dietary treatments: fed a basal layer diet supplemented with 0.14% L-Lys-HCl and 0.17% DL-Met (control) and the basal diet supplemented with encapsulated Lys and Met at the level of 60% (60CLM) or 80% of control (80CLM), respectively. The basal diet was formulated according to the recommendations of the National Research Council standard (NRC, 1994, Table 1). The experiment lasted 8 wk. Three hens were raised in an individual battery cage (60 cm length $\times$ 45 cm width $\times$ 50 cm height), and each hen had approximately 900 cm² of floor space. Housing temperature and relative humidity were maintained at 23 ± 2 °C and $65\% \pm 3\%$, respectively. The photoperiod was 16:8 (L:D) h. Each cage was equipped with one nipple drinker and a feeder. All hens had free access to feed and water throughout the experimental period.

The BW of each hen was recorded at the beginning and end of the experiment. Egg number and weight were recorded daily, and feed intake was recorded weekly. Feed intake, laying rate, and egg weight were calculated based on the collected data. Egg mass (g/hen/d) was calculated by multiplying average egg weight by laying rate, and feed efficiency was calculated as gram egg mass per day per hen divided by gram feed consumption per day per hen. In the fourth and eighth weeks, eggs were collected for three consecutive days and analyzed for egg quality.

At the end of week 4, four hens were randomly selected from each replicate ($n = 10$), such that each dietary treatment had 20 birds used for blood sampling. The blood sample was drawn

Table 1. Ingredient and nutrition composition of experimental diets (DM basis)

Ingredients, %	Control	80CLM ¹	60CLM ¹
Corn	64.18	63.99	64.12
Soybean meal, 45% CP	20.56	20.56	20.56
Rapeseed meal, 37% CP	2	2	2
Wheat bran	1	1	1
Soybean oil	0.91	0.91	0.91
Limestone	8.77	8.77	8.77
Dicalcium phosphate	1.62	1.62	1.62
Salt	0.31	0.31	0.31
Choline chloride	0.09	0.09	0.09
L-Lys-HCl (99%)	0.14	—	—
DL-Met (99%)	0.17	—	—
Encapsulated L-Lys-HCl	—	0.224	0.168
Encapsulated DL-Met	—	0.272	0.204
Premix ²	0.25	0.25	0.25
Calculated nutrition level			
ME, kcal/kg	2,700	2,700	2,700
Trp	0.2	0.2	0.2
Analyzed nutrition level			
CP, %	15.53	15.47	15.61
Ca, %	3.64	3.65	3.66
AP, %	0.45	0.46	0.44
Lys	0.84	0.80	0.75
Met	0.44	0.39	0.35
Met + Cys	0.64	0.61	0.58
Thr	0.55	0.58	0.55
Gly	0.45	0.45	0.45
Val	0.65	0.64	0.65
Ile	0.49	0.46	0.46
Leu	0.99	0.95	0.95
Phe	0.72	0.76	0.74
Arg	0.55	0.58	0.57
Asp	1.15	1.11	1.15
Ser	0.67	0.67	0.66
Glu	2.25	2.22	2.27
Ala	0.68	0.67	0.67
His	0.55	0.53	0.54

¹Basal diet supplemented with encapsulated L-lys HCL and DL-met at 60% (60 CLM) or 80% (80CLM) of the control levels.

²The vitamin and mineral premix provide the follow quantities per kilogram of diet: vitamin A, 8,800 IU; vitamin D3, 3,300 IU; vitamin K, 2.2 mg; vitamin E, 16.5 IU; cholecalciferol, 2,800 IU; riboflavin, 18 mg; niacin, 50 mg; pantothenic acid, 28 mg; biotin, 0.1 mg; folic acid, 0.6 mg; iron, 55 mg; selenium, 0.3 mg; copper, 5.5 mg; zinc, 88 mg; iodine, 1.7 mg; and manganese, 88 mg.

from the left-wing vein using 5 mL heparinized syringe (needle length: 3 cm) from each hen and collected at fed state (6 h after the light turned on) and fasting state (12-h overnight feed withdrawal). The blood sample was collected with ice-cold tube. Plasma samples were obtained after centrifugation at $3,000 \times g$ for 15 min at 4 °C and stored at -20 °C for further analysis.

At end of the experiment, two hens from each replicate were randomly selected for blood sampling ($n = 10$). After 12-h feed withdrawal, the blood sample was drawn from the left wing vein using 5 mL heparinized syringe (needle length: 3 cm) from each hen. The blood sample was collected with ice-cold tube. Plasma samples were obtained and stored at -20 °C for further analysis. After blood sampling, the bird was sacrificed by exsanguination after cervical dislocation. The small intestine was dissected from the mesentery and immediately placed on ice. Intestinal segments (2.5 cm in length) of the

duodenum, jejunum, and ileum were obtained and fixed in 4% neutral-buffered formalin for future histological analysis. The intestinal mucosa was obtained in the duodenum, jejunum, and ileum, respectively. The mucosa samples were snap-frozen in liquid nitrogen and then stored at -80 °C for further analysis.

Experiment 2

Twenty-four 55-wk-old Hy-Line Brown laying hens, of similar BW (2.02 ± 0.04 kg), were randomly divided into three groups of eight hens and reared individually in battery cage (60 cm length $\times$ 45 cm width $\times$ 50 cm height). The experimental hens were provided with meal feeding (40 g feed within 30 min) three times a day and trained to consume each meal within 30 min. After 12-h feed withdrawal, each hens was, respectively, offered 30 g of the aforementioned three diets described in exp. 1. A blood sample was collected from all of the hens via left-wing vein at 2, 4, and 6 h after the meal, respectively. The method of blood collection was the same as the exp. 1. Plasma samples were obtained after centrifugation at $3,000 \times g$ 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 (DM; method 930.15), crude protein (CP; method 990.03), calcium (Ca; method 984.01), and phosphorus (AP; method 965.17) of basal diet as described by [AOAC International \(1996\)](#). Dietary AA were determined by ion-exchange chromatography with post-column ninhydrin detection using a Hitachi L-8900 AA Analyzer (Tokyo, Japan) after acid hydrolysis with 6 N HCl and reflux for 24 h. Methionine and cysteine were analyzed as Met sulfone and cysteic acid after cold performic acid oxidation overnight before hydrolysis.

Plasma parameters

Plasma samples were analyzed for alanine aminotransferase (ALT), aspartate aminotransferase (AST), total protein, urate, and urea nitrogen (urea-N) using the Hitachi L-7020 fully automatic biochemical analyzer (Tokyo, Japan). The autoanalyzer has validated for avian plasma samples. A total free amino acid (TAA) assay kit (AA-1-W, Keming, Suzhou, China) was used to determine the content of total free amino acid in plasma, according to the manufacturer's instructions. Plasma free AA concentrations were determined by ion-exchange chromatography using a Hitachi L-8900 AA Analyzer (Tokyo, Japan) under physiological fluid analysis conditions. Frozen plasma samples (800 μ L) were thawed at 4 °C and then deproteinized with 40 mg of sulfosalicylic acid. The sample was mixed by vortexing with an oscillator (Guohua Electric Appliance, Changzhou, China). After sitting for 4 h at 4 °C, the sample was then centrifuged at $12,000 \times g$ for 30 min. The supernatant fluid was collected and passed through a filter (0.1 μ m) before amino acid analysis.

Egg quality measurement

The egg shape index was calculated by dividing the egg width by the egg length. Eggshell thickness was measured by averaging the three locations on the egg (air cell, equator, and sharp end) using an eggshell thickness tester (ETG-1061, Tokyo, Japan). Eggshell breaking strength was measured using an egg force reader (EFG-0503, Tokyo, Japan). Yolk color, Haugh unit, and the height of albumen were measured using the egg equality analyzer (EMT-5200, Tokyo, Japan). Yolk and albumen were separated and weighed as indicated by [Grobas et al. \(2001\)](#),

Table 2. Primers used for real-time quantitative PCR

Gene	Genbank accession no.	Orientation	Sequences (5'-3')	Product size, bp
CAT-1	NM_001145490.1	Forward	GCTCTATGGTGTGGAGGG	192
		Reverse	AATAAGCCACAAAGCAGATGAG	
b ⁰ -AT	NM_001199133.1	Forward	TGTGTTGCTCTCTAACTGGCTG	154
		Reverse	CCTCCTTTCTGTTGCTCTGTTT	
y ⁺ LAT1	XM_418326.5	Forward	CATTCTCAGGGTTTCAGAGCAC	216
		Reverse	CTGTCCCTTTCTCCCATCGTG	
rBAT	XM_004935370.2	Forward	TTGGCTTGGCAAAGGAGTC	146
		Reverse	TCGGAATAGGCTGTGATGCT	
B ⁰ AT	XM_419056	Forward	AATGGGACAACAAGGCTCAG	125
		Reverse	CAAGATGAAGCAGGGGGATA	
EAAT3	XM_424930	Forward	ACCCTTTTGCCTTGGAACT	122
		Reverse	TTGAGATGTTTGCCTGAAG	
PepT1	NM_204365	Forward	ACACGTTTGTGCTCTGTGC	122
		Reverse	GACTGCCTGCCCAATTGTAT	
β-Actin	XM_003357928	Forward	TGCGGGACATCAAGGAGAAG	216
		Reverse	AGTTGAAGGTGGTCTCGTGG	

Table 3. Effect of encapsulated L-Lys-HCl and DL-Met supplementation on the laying performance of hens^{1,2}

Item	Control	80CLM	60CLM	P-value
Final BW, kg	2.07 ± 0.02	2.12 ± 0.07	2.08 ± 0.08	0.858
Feed intake, g/d	128.2 ± 2.2	132.2 ± 2.9	126.3 ± 3.1	0.327
Laying rate, %	87.1 ± 1.1	87.4 ± 0.8	85.8 ± 1.2	0.172
Egg mass, g/hen/d	56.6 ± 0.8	57.2 ± 0.6	56.2 ± 0.8	0.293
Egg weight, g	65.0 ± 0.25	65.5 ± 0.16	65.5 ± 0.20	0.168
Feed efficiency, g/g	0.44 ± 0.03	0.43 ± 0.05	0.44 ± 0.05	0.761

¹Basal diet supplemented with encapsulated L-lys HCl and DL-met at 60% (60 CLM) or 80% (80CLM) of the control levels.

²Data were presented as the mean ± SEM (n = 5).

and their relative proportions (% egg weight) were determined. Eggshell was weighed after drying 12 h at room temperature.

Intestinal histological analysis

Intestinal segments were embedded in paraffin and cut into 5-μm serial sections. Three histochemical 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 Analyzer (Lucia Software, Lucia, Za Drahov, Czechia). The 18 measurements were averaged to yield 1 value per laying hen. These procedures were conducted by an observer unaware of the dietary treatments.

RNA isolation and real-time polymerase chain reaction analysis

Total ribonucleic acid (RNA) was isolated from a sample of each intestinal segment (~100 mg, frozen in liquid nitrogen) using TRIzol reagents (Invitrogen, USA). RNA quality was determined with agarose gel electrophoresis and a spectrophotometry (Eppendorf, Germany) detecting the UV absorbance ratio at 260 and 280 nm (A260/280 ≈ 1.75 to 2.01). Then, 1 μg of total RNA was reverse-transcribed to complementary deoxyribose nucleic acid (cDNA) using DNase I (Invitrogen, USA) according to the manufacturer's protocol. Primers were designed with the use of DNAMAN software on the basis of chicken gene sequences (Table 2); β-actin was used as an internal reference gene to

normalize target gene transcript level. Real-time polymerase chain reaction (RT-PCR) was performed using an Applied Biosystems Quantstudio 5 Real-time PCR system (Foster City, USA). The system included 2 μL DNA template, 10 μL SYBR Green mix (containing MgCl₂, deoxy-ribonucleoside triphosphate, and Hotstar Taq polymerase), 7 μL deionized H₂O, and 0.5 μL each of forward and reverse primers, for a total volume of 20 μL. The protocols included pre-denaturation (10 s at 95 °C), amplification, and quantification program repeated for 40 cycles (5 s at 95 °C and 34 s at 60 °C). A standard curve and a melt curve were plotted to calculate the efficiency of the primers. Eight samples were used for each treatment, and every sample was measured in triplicate. The relative expression of genes was compared with the control group using the cycle threshold (Ct) values (Fu et al., 2006).

Statistical analysis

Prior to analysis, all data were examined for the homogeneity and normal distribution plots of variances among the treatments by using UNIVARIATE procedure. Statistical analysis was performed using SAS statistical software (SAS version 8.1; SAS Institute Inc., Cary, NC, USA). In exp. 1, for the laying performance and egg quality, a one-way ANOVA model was used to estimate the main effect of encapsulated L-Lys-HCl and DL-Met with each group as replicate. For the plasma parameters, intestinal morphology, and mRNA levels of AA transporters, a one-way ANOVA model was used to estimate the main effect of dietary treatment with individual hen as replicate. When the main effect of the treatment was significant, the differences between means were assessed by Tukey's multiple comparisons test. In exp. 2, the repeated measurement analysis was conducted to estimate the main effects of diet, time, and their interactions with each chicken as replicate. *P* < 0.05 was considered to be statistically significant.

Results

Experiment 1

No significant difference (*P* > 0.05) was observed in the final BW, feed intake, laying rate, egg mass, egg weight, or feed efficiency among the control, 60CLM, and 80CLM treatments; however,

Table 4. Effect of encapsulated L-Lys-HCl and DL-Met supplementation on egg quality^{1,2}

Item	Control	80CLM	60CLM	P-value
Week 4				
Egg weight, g	64.2 ± 0.9	65.2 ± 0.8	65.3 ± 0.7	0.591
Egg shape index, %	76.3 ± 0.3	75.3 ± 0.3	75.2 ± 0.5	0.193
Eggshell thickness, ×10 ² mm	32.8 ± 0.3	32.3 ± 0.9	32.7 ± 0.5	0.825
Eggshell strength, kg/cm ²	4.1 ± 0.1	4.2 ± 0.1	4.1 ± 0.1	0.934
Albumen height, mm	6.4 ± 0.3	6.5 ± 0.2	6.3 ± 0.2	0.775
Yolk color score	6.2 ± 0.2	6.7 ± 0.4	6.2 ± 0.4	0.454
Haugh units	77.9 ± 1.9	77.7 ± 1.8	76.2 ± 1.8	0.779
Yolk, %	26.2 ± 0.4	26.5 ± 0.3	26.2 ± 0.3	0.802
Albumen, %	64.1 ± 0.5	64.1 ± 0.3	63.6 ± 0.6	0.670
Eggshell, %	9.6 ± 0.1	9.5 ± 0.2	10.2 ± 0.4	0.170
Week 8				
Egg weight, g	65.5 ± 0.8	65.6 ± 0.4	66.4 ± 0.7	0.624
Egg shape index, %	75.4 ± 0.6	75.7 ± 0.5	74.4 ± 0.4	0.226
Eggshell thickness, ×10 ² mm	32.8 ± 0.5	33.1 ± 0.4	33.8 ± 0.5	0.186
Eggshell strength, kg/cm ²	4.1 ± 0.1 ^a	4.1 ± 0.1 ^a	3.8 ± 0.1 ^b	0.031
Albumen height, mm	6.9 ± 0.2	7.0 ± 0.2	6.7 ± 0.1	0.508
Yolk color score	7.2 ± 0.2	7.1 ± 0.3	7.0 ± 0.2	0.804
Haugh units	79.9 ± 1.4	81.5 ± 1.3	79.2 ± 0.5	0.368
Yolk, %	26.2 ± 0.5	26.3 ± 0.3	26.7 ± 0.3	0.706
Albumen, %	63.6 ± 0.6	63.5 ± 0.3	63.2 ± 0.4	0.813
Eggshell, %	10.1 ± 0.2	10.3 ± 0.1	10.1 ± 0.1	0.777

¹Basal diet supplemented with encapsulated L-lys HCL and DL-met at 60% (60 CLM) or 80% (80CLM) of the control levels.

²Data were presented as the mean ± SEM (n = 5).

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

Table 5. Effects of encapsulated L-Lys-HCl and DL-Met supplementation on plasma concentrations of ALT, AST, total protein, urate, urea-N, and TAA at the fourth and eighth week^{1,2}

Item	Control	80CLM	60CLM	P-value
Week 4: fed state				
ALT, U/L	8.09 ± 0.16 ^b	8.87 ± 0.31 ^a	8.68 ± 0.18 ^{ab}	0.045
AST, U/L	149 ± 5.0	163 ± 6.0	159 ± 4.4	0.182
Total protein, μmol/L	52.7 ± 1.9	49.3 ± 2.5	56.0 ± 2.2	0.120
Urate, μmol/L	237 ± 22.5	224 ± 17.1	246 ± 9.6	0.657
Urea N, mmol/L	0.30 ± 0.03	0.31 ± 0.03	0.35 ± 0.02	0.487
TAA, μmol/mL	9.52 ± 0.68	10.41 ± 0.96	10.67 ± 0.99	0.659
Week 4: fasting state				
ALT, U/L	8.21 ± 0.32	8.23 ± 0.26	9.02 ± 0.69	0.386
AST, U/L	155 ± 8.6	150 ± 2.9	173 ± 10.0	0.126
Total protein, μmol/L	46.3 ± 1.98	48.4 ± 1.2	46.2 ± 1.1	0.507
Urate, μmol/L	158 ± 14.0	165 ± 26.7	152 ± 22.8	0.311
Urea N, mmol/L	0.18 ± 0.03	0.19 ± 0.02	0.17 ± 0.03	0.808
TAA, μmol/mL	8.93 ± 1.04	9.43 ± 0.84	8.70 ± 0.53	0.822
Week 8: fasting state				
ALT, U/L	8.94 ± 0.45	8.44 ± 0.39	8.83 ± 0.15	0.584
AST, U/L	137 ± 7.0	143 ± 7.6	154 ± 4.2	0.185
Total protein, μmol/L	52.0 ± 2.2	52.0 ± 1.6	53.1 ± 2.3	0.911
Urate, μmol/L	187 ± 21.4	175 ± 18.6	192 ± 31.1	0.513
Urea N, mmol/L	0.34 ± 0.04	0.31 ± 0.03	0.26 ± 0.02	0.214
TAA, μmol/mL	10.2 ± 1.10	11.6 ± 1.00	9.8 ± 1.28	0.311

¹Basal diet supplemented with encapsulated L-lys HCL and DL-met at 60% (60 CLM) or 80% (80CLM) of the control levels.

²Data were presented as the mean ± SD (n = 10).

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

60CLM diet had lower feed intake, laying rate, and egg mass values than in control and 80CLM treatments (Table 3). At week 4, dietary treatment had no significant influence on egg shape index, eggshell thickness, eggshell strength, albumen height and percentage, yolk color, or Haugh unit (P > 0.05, Table 4). At the end of the experiment, none of the egg quality parameters were changed by dietary treatments, except that the eggshell strength was reduced (P < 0.05) by 60CLM treatment, compared with the control and 80CLM treatments (Table 4).

After 4 wk of treatment, plasma AST activity, total protein, urate, urea N, and TAA levels were not changed (P > 0.05) by dietary treatment at either fed or fasting state (Table 5). The ALT activity, however, was elevated under 80CLM treatment at fed state, compared with control hens (P < 0.05). At week 8, none of the plasma parameters measured at fasting state were influenced by dietary treatment (P > 0.05, Table 5).

Villus height and crypt depth in the duodenum, jejunum, and ileum were not influenced by dietary treatment (P > 0.05, Table 6). However, the villus height to crypt depth ratio in the duodenum was lower in 80CLM-hens compared with control hens (P < 0.05). Dietary treatment had no influence on the villus height to crypt depth ratio in the jejunum or the ileum (P > 0.05).

The mRNA levels of AA transporters such as b^{0,+} amino acid transporter (b^{0,+}AT), cationic amino acid transporter-1 (CAT-1), y⁺L amino acid transporter-1 (y⁺LAT1), B⁰ neutral amino acid transporter (B⁰AT), related to b^{0,+} amino acid transporter (rBAT), acidic AA transporter (EAAT3), and intestinal peptide transporter-1 (PepT1) in the duodenum, jejunum, and ileum were not altered by dietary treatment (P > 0.05, Table 7).

Experiment 2

The postprandial metabolites were measured at 2, 4, and 6 h time points to evaluate the effect of dietary AA treatment on

Table 6. Effect of encapsulated L-Lys-HCl and DL-Met supplementation on intestinal villus height and crypt depth of laying hens^{1,2}

Item	Control	80CLM	60CLM	P-value
Duodenum				
Villus height, μm	1,615 $\pm$ 73.9	1,563 $\pm$ 126.9	1,504 $\pm$ 68.8	0.712
Crypt depth, μm	261.8 $\pm$ 18.3	334.3 $\pm$ 28.2	292.6 $\pm$ 27.6	0.165
Villus height: crypt depth	6.3 $\pm$ 0.4 ^a	4.7 $\pm$ 0.4 ^b	5.4 $\pm$ 0.5 ^{ab}	0.020
Jejunum				
Villus height, μm	1,249 $\pm$ 92.1	1,374 $\pm$ 131.1	1,325 $\pm$ 100.7	0.718
Crypt depth, μm	300.8 $\pm$ 16.5	278.4 $\pm$ 9.6	245.4 $\pm$ 25.5	0.132
Villus height: crypt depth	4.3 $\pm$ 0.6	5.0 $\pm$ 0.6	5.6 $\pm$ 0.6	0.364
Ileum				
Villus height, μm	991.6 $\pm$ 61.6	924.2 $\pm$ 82.7	1,098.0 $\pm$ 107.6	0.378
Crypt depth, μm	230.6 $\pm$ 23.8	203.1 $\pm$ 27.5	208.6 $\pm$ 22.6	0.712
Villus height: crypt depth	4.5 $\pm$ 0.5	4.9 $\pm$ 0.6	5.6 $\pm$ 0.9	0.509

¹Basal diet supplemented with encapsulated L-lys HCL and DL-met at 60% (60 CLM) or 80% (80CLM) of the control levels.²Data were presented as the mean $\pm$ SD ($n = 6$).^{a,b}Means with different superscripts within the same line differ significantly, $P < 0.05$.**Table 7.** Effect of encapsulated L-Lys-HCl and DL-Met supplementation on the gene expression of amino acid transporters in different sections of intestinal tract^{1,2}

Item	Control	80CLM	60CLM	P-value
Duodenum				
b ⁰ +AT	1.00 $\pm$ 0.07	1.05 $\pm$ 0.12	1.12 $\pm$ 0.13	0.756
CAT-1	1.00 $\pm$ 0.16	0.94 $\pm$ 0.09	0.92 $\pm$ 0.11	0.900
y ⁺ LAT1	1.00 $\pm$ 0.24	1.15 $\pm$ 0.18	0.90 $\pm$ 0.16	0.657
B ⁰ AT	1.00 $\pm$ 0.19	0.74 $\pm$ 0.15	0.72 $\pm$ 0.14	0.396
rBAT	1.00 $\pm$ 0.14	0.75 $\pm$ 0.07	0.80 $\pm$ 0.06	0.177
EAAT3	1.00 $\pm$ 0.11	0.86 $\pm$ 0.06	0.97 $\pm$ 0.11	0.581
PepT1	1.00 $\pm$ 0.14	0.80 $\pm$ 0.10	1.06 $\pm$ 0.16	0.306
Jejunum				
b ⁰ +AT	1.00 $\pm$ 0.21	0.74 $\pm$ 0.09	0.91 $\pm$ 0.14	0.491
CAT-1	1.00 $\pm$ 0.12	1.06 $\pm$ 0.14	0.92 $\pm$ 0.09	0.728
y ⁺ LAT1	1.00 $\pm$ 0.17	1.15 $\pm$ 0.19	1.31 $\pm$ 0.23	0.548
B ⁰ AT	1.00 $\pm$ 0.18	1.10 $\pm$ 0.21	0.93 $\pm$ 0.17	0.816
rBAT	1.00 $\pm$ 0.11	0.80 $\pm$ 0.10	0.94 $\pm$ 0.24	0.665
EAAT3	1.00 $\pm$ 0.22	0.67 $\pm$ 0.09	0.73 $\pm$ 0.09	0.260
PepT1	1.00 $\pm$ 0.12	0.56 $\pm$ 0.12	0.94 $\pm$ 0.19	0.087
Ileum				
b ⁰ +AT	1.00 $\pm$ 0.08	1.00 $\pm$ 0.10	0.90 $\pm$ 0.19	0.813
CAT-1	1.00 $\pm$ 0.13	1.14 $\pm$ 0.21	1.01 $\pm$ 0.18	0.882
y ⁺ LAT1	1.00 $\pm$ 0.23	1.08 $\pm$ 0.24	0.80 $\pm$ 0.09	0.545
B ⁰ AT	1.00 $\pm$ 0.16	1.19 $\pm$ 0.19	0.99 $\pm$ 0.16	0.697
rBAT	1.00 $\pm$ 0.09	0.98 $\pm$ 0.14	1.02 $\pm$ 0.16	0.979
EAAT3	1.00 $\pm$ 0.11	0.91 $\pm$ 0.21	0.96 $\pm$ 0.19	0.932
PepT1	1.00 $\pm$ 0.10	1.25 $\pm$ 0.13	1.04 $\pm$ 0.16	0.412

¹Basal diet supplemented with encapsulated L-lys HCL and DL-met at 60% (60 CLM) or 80% (80CLM) of the control levels.²Data were presented as the mean $\pm$ SD ($n = 8$).

AA metabolism. Dietary AA treatment had no detectable effect ($P > 0.05$), while time had a significant influence on plasma urate, total indispensable amino acids (TIAA), total dispensable amino acids (TDAA), and TAA ($P < 0.001$, Figure 1). There was a significant interaction of dietary AA treatment and time on TDAA ($P < 0.05$, Figure 1C). The 80CLM group exhibited lower TDAA concentration compared with control hens at 6-h time point ($P < 0.05$, Figure 1C), but not at the 2- and 4-h time points ($P > 0.05$).

Time had a significant influence on Lys, Arg, Thr, Leu, Ile, His, Phe, Val, and Gly ($P < 0.001$) whereas had an influence on Ser

($P > 0.05$, Figure 2). Dietary AA treatment had a significant influence on Ser ($P < 0.05$) and Phe ($P < 0.05$). The control hens had the highest Ser level at 2- and 6-h time points compared with 60CLM and 80CLM birds ($P < 0.05$, Figure 2F). In contrast, the control hens had the highest and 60CLM hens had the lowest Phe at 2-h time point ($P < 0.05$, Figure 2H). There was an interaction of time and dietary AA treatment on Lys ($P < 0.05$). Dietary AA treatment showed a trend to take an effect on Lys ($P = 0.073$, Figure 2A) only at 2-h time point, and the control hens had the lowest Lys level compared with 60CLM and 80CLM birds.

There was a time effect on Met ($P < 0.05$) and Tau ($P < 0.001$, Figure 3A and C), whereas Cys showed a trend to be changed by time ($P = 0.069$, Figure 3B). There was no interaction of time and dietary AA treatment on Met, Cys, and Tau ($P > 0.05$). Dietary AA treatment had a significant influence on Cys and Tau ($P < 0.01$) but had no effect on Met ($P > 0.05$, Figure 3A). The 60 CLM hens had lower Cys level at 2-, 4-, and 6-h time points compared with control hens ($P < 0.05$, Figure 3B), while the Cys in 80CLM was lowered at 6-h time point compared with control ($P < 0.05$). In contrast, the Tau level was higher in 80CLM-hens at 2- and 4-h time points ($P < 0.05$, Figure 3C), and there was no difference between control and 60CLM groups ($P > 0.05$).

Discussion

These results demonstrate that supplementation with encapsulated L-Lys-HCl and DL-Met at 80% of control levels did not negatively affect laying hen performance. This finding suggests that encapsulated AA could reduce the supplemental level of crystalline AA by ameliorating postabsorptive AA balance in laying hens.

Met and Lys are, respectively, the first- and second-limiting AA in corn-soybean diets for laying hens. Increasing the digestible Lys level had significant effects on egg production, egg weight, egg mass, and feed efficiency (Spangler et al., 2019). Supplementation with L-Lys-HCl and DL-Met sustains the laying performance of hens on a relatively low-protein diet (Schutte et al., 1983). In contrast, feeding excesses of individual AA, such as Lys, Met, Thr, or Trp, in a practical layer diet (16% CP corn and soybean meal) had no significant beneficial effect on laying performance (Koelkebeck et al., 1991). In the present study, the laying rate, egg weight, egg mass, feed intake, and feed efficiency were not significantly changed by 60CLM and 80CLM treatments,

Figure 1. Effects of encapsulated L-Lys-HCl and DL-Met supplementation on postprandial plasma urate (A), TIAA (B), TDAA (C), and TAA (D) concentrations ($\mu\text{g/mL}$) at 2-, 4-, and 6-h time points. Data are shown as the mean $\pm$ SD ($n = 8$). ^{a,b}Means with different letters within the same postprandial time point differ significantly, $P < 0.05$.

suggesting that the dietary Met and Lys levels are adequate for the laying hens. The result was in line with the observation that the BW of hens during the experimental period was not significantly changed by dietary AA treatments. However, this result should be explained with caution, it is also possible that body composition may be different among treatment groups. Furthermore, there were no significant differences in plasma urate level or TAA among control, 60CLM, and 80CLM treatments at either fed or fasting states. Urate is the main end product of nitrogen metabolism in birds. Serum urate can be used as an indicator of AA utilization in broilers fed AA-adequate and AA-deficient diets (Donsbough et al., 2010). Hence, the result suggests that the application of encapsulated Lys + Met has no disadvantage influence on the laying performance of hens.

In this study, however, eggshell strength was lower in the 60CLM group than in control and 80CLM treatments. The sulfur AA play a role in eggshell quality. Increasing the sulfate groups in the shell protein matrix increases the Ca-binding ability, which, in turn, increases both shell strength and shell percentage, resulting in improved overall shell quality (Novak et al., 2004). Under high ambient temperature, the sulfur amino acid requirement for shell protein matrix synthesis needs to be considered to optimize shell quality (Torki et al., 2015). In contrast, the influence of dietary sulfur AA on eggshell quality depends on the age of layers. Dietary Met + Cys level had a linear

influence on the eggshell thickness of Lohmann layers at 34 wk of age but had a quadratic effect on eggshell percentage and thickness of 50-wk-old layers (Carvalho et al., 2018). Dynamic feeding also has an effect on eggshell quality. Feeding low Met in the morning and high Met in the afternoon improved eggshell thickness (Liu et al., 2017). Hence, the depressed eggshell strength observed with the 60CLM diet may have resulted from reduced dietary Met level in 60CLM hens.

Dietary AAs are essential precursors for the intestinal synthesis of proteins and maintaining small intestinal integrity, as well as function (Bergen and Wu, 2009; Qiu et al., 2016). Villus height and crypt depth are important factors that influence nutrient exchange area for digestion and absorption (Pluske et al., 1996). In the current study, neither villus height nor crypt depth was influenced by 60CLM or 80CLM treatments compared with the control group, suggesting that replacing crystalline Lys and Met with encapsulated ones has no disadvantageous effect on the histomorphology of the intestinal tract. In piglets, small intestinal morphology would be damaged when fed a lysine-deficient diet (He et al., 2013). Hence, the present result indicates that encapsulated Lys + Met at 60% and 80% of control levels is adequate for the maintenance of gut morphology.

The small intestine is the main location for protein digestion and AA absorption. We further measured the gene expression of AA transporters in the small intestine.

Figure 2. Effects of encapsulated L-Lys-HCl and DL-Met supplementation on postprandial plasma lysine (A), arginine (B), threonine (C), leucine (D), isoleucine (E), serine (F), histidine (G), phenylalanine (H), valine (I), and glycine (J) concentrations ($\mu\text{g/mL}$) at 2-, 4-, and 6-h time points. Data are shown as the mean $\pm$ SD ($n = 8$). ^{a,b}Means with different letters within the same postprandial time point differ significantly, $P < 0.05$.

AA are transported into the cell in the free form by specific transporters (Leibach and Ganapathy, 1996; Kanai and Hediger, 2003; Wang et al., 2009). Dietary AA levels can affect AA transport by the gut (Wolfram et al., 1984; Stein et al., 1987). A reduction in AA intake increases the small intestinal *PepT1* abundance in rats (Ihara et al., 2000). The expression of cationic AA transporter *b⁰+AT* in the duodenum and *CAT-1*

in the jejunum can be enhanced by increased dietary levels of free lysine and other crystalline AA (Yin et al., 2014). In the present study, the expression of *CAT-1*, *y⁺LAT1*, *b⁰+AT*, *B⁰AT*, *rBAT*, *EAAT3*, and *PepT1* in the duodenum, jejunum, and ileum was not influenced by dietary treatment, suggesting that supplementation with encapsulated Lys and Met has no influence on AA transport.

Figure 3. Effects of encapsulated L-Lys-HCl and DL-Met supplementation on postprandial plasma methionine (A), cystine (B), and taurine (C) concentrations ($\mu\text{g/mL}$) at 2-, 4-, and 6-h time points. Data are shown as the mean $\pm$ SD ($n = 8$). ^{a,b}Means with different letters within the same postprandial time point differ significantly, $P < 0.05$.

The plasma concentrations of free AA at 2, 4, and 6 h after feeding were measured to further evaluate the effect of dietary supplementation with encapsulated Met + Lys on the dynamic utilization and metabolism of AA. Plasma free AA pool comprises of the AA absorbed from the digestive tract and the AA mobilized from body protein and serves principally as the direct source of AA supply for protein biosynthesis. The plasma concentration of the limiting AA can serve as a signal for dietary AA deficiency (Gloaguen et al., 2012). In the present study, there were no differences in plasma TIAA and TAA among control, 60CLM, and 80CLM hens. In contrast, the control hens had higher TDAA ($P < 0.05$) at the 6-h time point than did 80CLM hens. The result implies that dietary supplementation of encapsulated Met + Lys has an influence on the composition of blood free AA pool.

Compared with AA derived from proteins, crystalline AA are absorbed fast in the gut. In a previous study of pigs, the portal and arterial plasma lysine concentrations attained maximal levels by 1 h postprandial with a 12% CP + AA diet, but peaked at 2.5 h postprandial when the 16% CP diet was given (Yen et al., 2004). In the present study, although the control diet had 20% and 40% more crystalline L-Lys-HCl and DL-Met compared with the 80CLM and 60CLM diets, the 80CLM hens exhibited higher plasma Lys levels at the 2-h time point compared with control hens, suggesting two possibilities: increased Lys clearance and/or earlier postprandial peak level in control hens compared with 80CLM hens. This result was consistent with

the dynamic change in plasma Lys concentration in the control group and in 60CLM and 80CLM treatments, which has no detectable difference at 4- and 6-h time points. Taken together, these results suggest that encapsulated Lys may delay the Lys absorption.

In this study, plasma Met level was not changed by dietary AA treatments at the three time points, whereas Cys was reduced in 80CLM and 60CLM treatments. In contrast, taurine, the product of sulfur amino acid metabolism, was higher in 80CLM and 60CLM treatments ($P < 0.05$). As an indispensable amino acid, Met has important metabolic functions and forms Cys by *trans*-sulfuration (Xie et al., 2004; Martínvenegas et al., 2006). Taurine is synthesized from Cys and is involved in many physiological functions, including osmoregulation, detoxification, and antioxidation (Roig-Pérez et al., 2005). The relative lower Cys level in 60CLM and 80CLM birds may be a result of increased formation of taurine. Dietary taurine addition improves the performance of chicks fed a purified diet deficient in sulfur AA (Anderson et al., 1975). The present result indicated that dietary supplementation with encapsulated Met + Lys changes plasma sulfur AA profile with elevated Tau and decreased Cys. The role of taurine in the maintenance of laying performance of hen, however, remains to be elucidated in future research.

Compared with control, the relative lower Ser in 60CLM and 80CLM at 2-h time point and especially at 6-h time point indicated the reduced availability of Ser by encapsulated Met + Lys treatment.

It is reported that there is a linear increase in Ser concentration as dietary DL-Met supplementation level increased in broiler breeder hens (Wan et al., 2017). In broilers, the requirement for Gly and Ser is reduced when both Met and Cys are supplied adequately (Siebert et al., 2015). Hence, the result suggests that the reduced plasma level of Ser may be a direct effect of reduced dietary DL-Met supply. Moreover, Met and Cys are the key components of one-carbon metabolism (Kalhan and Marczewski, 2012), while Ser and Gly are the main sources of one-carbon groups (Locasale, 2013). The folate cycle and the Met cycle comprise of two metabolic pathways of one-carbon metabolism, and homocysteine can accept the carbon from the folate pool through 5-methyltetrahydrofolate to generate Met (Locasale, 2013). Hence, the result suggests that one-carbon metabolism may be involved in the treatment of encapsulated DL-Met in laying hens, and the underlying mechanism needs to be investigated further.

Compared with control hens, the changed plasma Phe levels in the 60CLM and 80CLM treatments at the 2-h time point indicated the altered balance of AA. This result was consistent with previous work by Dahiya et al. (2007), who reported that plasma free AA profile was changed by dietary Met source and level, and implies that altered crystalline DL-Met and L-Lys absorption changes AA metabolism in laying hens. The modified postabsorptive AA balance should be responsible for the maintenance of laying performance.

Supplemental levels of L-Lys-HCl and DL-Met in 60CLM and 80CLM treatments were 60% and 80% of control levels, respectively. Hence, the present results suggest that encapsulated Lys + Met can reduce the supplemental levels of synthetic AA. This finding is consistent with the previous study of pigs by Prandini et al. (2013), who reported that the use of microencapsulated Lys, compared with both crystalline L-Lys-HCl and dietary protein-bound Lys, can save CP and synthetic AA in diet formulation. Collectively, the present results demonstrate that encapsulated L-Lys-HCl and DL-Met change the profiles of blood-free AA in laying hens and decrease the supplemental level of synthetic L-Lys-HCl and DL-Met.

In conclusion, the results demonstrate that the supplemental level of synthetic L-Lys-HCl and DL-Met in encapsulated form can effectively decrease their supplemental level by approximately 20% without any negative influence on laying performance. The result also suggests that the improved postabsorptive AA balance contributes to the reduced dietary AA supplemental levels.

This work was supported by the Shangdong Key Research and Development Program (2018GNC111008), the National Key Research Program of China (2016YFD0500510), the China Agriculture Research System (CARS-40-K14), the National Natural Science Foundation of China (31772619), and the Taishan Scholars Program (201511023). We greatly thank the reviewers for their valuable comments and suggestions to the paper.

Conflict of interest statement

The authors declare no conflicts of interest.

Literature Cited

- Anderson, J. O., R. E. Warnick, and R. K. Dalai. 1975. Replacing dietary methionine and cystine in chick diets with sulfate or other sulfur compounds. *Poult. Sci.* 54:1122–1128. doi:10.3382/ps.0541122
- AOAC International. 1996. *Official methods of analysis*. 17th ed. Gaithersburg (MD): Association of Official Analytical Chemists.
- Baker, D. H. 2009. Advances in protein-amino acid nutrition of poultry. *Amino Acids* 37:29–41. doi:10.1007/s00726-008-0198-3
- Bergen, W. G., and G. Wu. 2009. Intestinal nitrogen recycling and utilization in health and disease. *J. Nutr.* 139:821–825. doi:10.3945/jn.109.104497
- Boirie, Y., M. Dangin, P. Gachon, M. P. Vasson, J. L. Maubois, and B. Beaufrère. 1997. Slow and fast dietary proteins differently modulate postprandial protein accretion. *Proc. Natl. Acad. Sci. U. S. A.* 94:14930–14935. doi:10.1073/pnas.94.26.14930
- Carvalho, T. S. M., L. S. Sousa, F. A. Nogueira, D. P. Vaz, M. M. Saldanha, M. V. Triginelli, M. F. V. S. Pinto, N. C. Baião, and L. J. C. Lara. 2018. Digestible methionine+cysteine in the diet of commercial layers and its influence on the performance, quality, and amino acid profile of eggs and economic evaluation. *Poult. Sci.* 97:2044–2052. doi:10.3382/ps/pey036
- Chow, J. M., E. J. DePeters, and R. L. Baldwin. 1990. Effect of rumen-protected methionine and lysine on casein in milk when diets high in fat or concentrate are fed. *J. Dairy Sci.* 73:1051–1061. doi:10.3168/jds.S0022-0302(90)78764-4
- Dahiya, J. P., D. Hoehler, A. G. Van Kessel, and M. D. Drew. 2007. Effect of different dietary methionine sources on intestinal microbial populations in broiler chickens. *Poult. Sci.* 86:2358–2366. doi:10.3382/ps.2007-00133
- Donsbough, A. L., S. Powell, A. Waguespack, T. D. Bidner, and L. L. Southern. 2010. Uric acid, urea, and ammonia concentrations in serum and uric acid concentration in excreta as indicators of amino acid utilization in diets for broilers. *Poult. Sci.* 89:287–294. doi:10.3382/ps.2009-00401
- Elango, R., R. O. Ball, and P. B. Pencharz. 2009. Amino acid requirements in humans: with a special emphasis on the metabolic availability of amino acids. *Amino Acids* 37:19–27. doi:10.1007/s00726-009-0234-y
- Fu, W. J., J. Hu, T. Spencer, R. Carroll, and G. Wu. 2006. Statistical models in assessing fold change of gene expression in real-time RT-PCR experiments. *Comput. Biol. Chem.* 30:21–26. doi:10.1016/j.compbiolchem.2005.10.005
- Choreyshi, S. M., B. Omri, R. Chalghoumi, M. Bouyeh, A. Seidavi, M. Dadashbeiki, M. Lucarini, A. Durazzo, R. van den Hoven, and A. Santini. 2019. Effects of dietary supplementation of L-carnitine and excess lysine-methionine on growth performance, carcass characteristics, and immunity markers of broiler chicken. *Animals* 9:362. doi:10.3390/ani9090608
- Gloaguen, M., N. Le Floch, E. Corrent, Y. Primot, and J. van Milgen. 2012. Providing a diet deficient in valine but with excess leucine results in a rapid decrease in feed intake and modifies the postprandial plasma amino acid and α -keto acid concentrations in pigs. *J. Anim. Sci.* 90:3135–3142. doi:10.2527/jas.2011-4956
- Grobos, S., J. Méndez, R. Lázaro, C. de Blas, and G. G. Mateo. 2001. Influence of source and percentage of fat added to diet on performance and fatty acid composition of egg yolks of two strains of laying hens. *Poult. Sci.* 80:1171–1179. doi:10.1093/ps/80.8.1171
- Han, Y., and D. H. Baker. 1993. Effects of excess methionine or lysine for broilers fed a corn-soybean meal diet. *Poult. Sci.* 72:1070–1074. doi:10.3382/ps.0721070
- He, L., H. Yang, Y. Hou, T. Li, J. Fang, X. Zhou, Y. Yin, L. Wu, M. Nyachoti, and G. Wu. 2013. Effects of dietary L-lysine intake on the intestinal mucosa and expression of CAT genes in weaned piglets. *Amino Acids* 45:383–391. doi:10.1007/s00726-013-1514-0
- Hiramoto, K., T. Muramatsu, and J. Okumura. 1990. Protein synthesis in tissues and in the whole body of laying hens during egg formation. *Poult. Sci.* 69:264–269. doi:10.3382/ps.0690264

- Ihara, T., T. Tsujikawa, Y. Fujiyama, and T. Bamba. 2000. Regulation of PepT1 peptide transporter expression in the rat small intestine under malnourished conditions. *Digestion* 61:59–67. doi:[10.1159/000007736](https://doi.org/10.1159/000007736)
- Kalhan, S. C., and S. E. Marczewski. 2012. Methionine, homocysteine, one carbon metabolism and fetal growth. *Rev. Endocr. Metab. Disord.* 13:109–119. doi:[10.1007/s11154-012-9215-7](https://doi.org/10.1007/s11154-012-9215-7)
- Kanai, Y., and M. A. Hediger. 2003. The glutamate and neutral amino acid transporter family: physiological and pharmacological implications. *Eur. J. Pharmacol.* 479:237–247. doi:[10.1016/j.ejphar.2003.08.073](https://doi.org/10.1016/j.ejphar.2003.08.073)
- Koelkebeck, K. W., D. H. Baker, Y. Han, and C. M. Parsons. 1991. Research Note: Effect of excess lysine, methionine, threonine, or tryptophan on production performance of laying hens. *Poult. Sci.* 70:1651–1653. doi:[10.3382/ps.0701651](https://doi.org/10.3382/ps.0701651)
- Leibach, F. H., and V. Ganapathy. 1996. Peptide transporters in the intestine and the kidney. *Annu. Rev. Nutr.* 16:99–119. doi:[10.1146/annurev.nu.16.070196.000531](https://doi.org/10.1146/annurev.nu.16.070196.000531)
- Li, Y., Z. Lv, Z. Li, L. Liu, F. Wang, D. Li, and C. Lai. 2018. Effects of feeding level and dietary supplementation with crystalline amino acids on digestible, metabolizable and net energy values of corn in growing pigs. *Anim. Feed Sci. Technol.* 240:197–205. doi:[10.1016/j.anifeedsci.2018.04.009](https://doi.org/10.1016/j.anifeedsci.2018.04.009)
- Liu, Y., X. Lin, X. Zhou, D. Wan, Z. Wang, X. Wu, and Y. Yin. 2017. Effects of dynamic feeding low and high methionine diets on egg quality traits in laying hens. *Poult. Sci.* 96:1459–1465. doi:[10.3382/ps/pew398](https://doi.org/10.3382/ps/pew398)
- Locasale, J. W. 2013. Serine, glycine and one-carbon units: cancer metabolism in full circle. *Nat. Rev. Cancer* 13:572–583. doi:[10.1038/nrc3557](https://doi.org/10.1038/nrc3557)
- Martívenegas, R., P. A. Geraert, and R. Ferrer. 2006. Conversion of the methionine hydroxy analogue DL-2-hydroxy-(4-methylthio) butanoic acid to sulfur-containing amino acids in the chicken small intestine. *Poult. Sci.* 85:1932–1938. doi:[10.1093/ps/85.11.1932](https://doi.org/10.1093/ps/85.11.1932)
- Mateo, R. D., G. Wu, H. K. Moon, J. A. Carroll, and S. W. Kim. 2008. Effects of dietary arginine supplementation during gestation and lactation on the performance of lactating primiparous sows and nursing piglets. *J. Anim. Sci.* 86:827–835. doi:[10.2527/jas.2007-0371](https://doi.org/10.2527/jas.2007-0371)
- Morales, A., L. Buenabad, G. Castillo, N. Arce, B. A. Araiza, J. K. Htoo, and M. Cervantes. 2015. Low-protein amino acid-supplemented diets for growing pigs: effect on expression of amino acid transporters, serum concentration, performance, and carcass composition. *J. Anim. Sci.* 93:2154–2164. doi:[10.2527/jas.2014-8834](https://doi.org/10.2527/jas.2014-8834)
- Novak, C., H. Yakout, and S. Scheideler. 2004. The combined effects of dietary lysine and total sulfur amino acid level on egg production parameters and egg components in Dekalb Delta laying hens. *Poult. Sci.* 83:977–984. doi:[10.1093/ps/83.6.977](https://doi.org/10.1093/ps/83.6.977)
- NRC. 1994. *Nutrient requirements of poultry*. 9th rev. ed. Washington (DC): National Academy Press.
- Overton, T. R., L. S. Emmert, and J. H. Clark. 1998. Effects of source of carbohydrate and protein and rumen-protected methionine on performance of cows. *J. Dairy. Sci.* 81:221–228. doi:[10.3168/jds.S0022-0302\(98\)75569-9](https://doi.org/10.3168/jds.S0022-0302(98)75569-9)
- Pennings, B., Y. Boirie, J. M. Senden, A. P. Gijzen, H. Kuipers, and L. J. van Loon. 2011. Whey protein stimulates postprandial muscle protein accretion more effectively than do casein and casein hydrolysate in older men. *Am. J. Clin. Nutr.* 93:997–1005. doi:[10.3945/ajcn.110.008102](https://doi.org/10.3945/ajcn.110.008102)
- Piva, A., V. Pizzamiglio, M. Morlacchini, M. Tedeschi, and G. Piva. 2007. Lipid microencapsulation allows slow release of organic acids and natural identical flavors along the swine intestine. *J. Anim. Sci.* 85:486–493. doi:[10.2527/jas.2006-323](https://doi.org/10.2527/jas.2006-323)
- Pluske, J. R., M. J. Thompson, C. S. Atwood, P. H. Bird, I. H. Williams, and P. E. Hartmann. 1996. Maintenance of villus height and crypt depth, and enhancement of disaccharide digestion and monosaccharide absorption, in piglets fed on cows' whole milk after weaning. *Br. J. Nutr.* 76:409–422. doi:[10.1079/bjn19960046](https://doi.org/10.1079/bjn19960046)
- Prandini, A., S. Sigolo, M. Morlacchini, E. Grilli, and L. Fiorentini. 2013. Microencapsulated lysine and low-protein diets: effects on performance, carcass characteristics and nitrogen excretion in heavy growing-finishing pigs. *J. Anim. Sci.* 91:4226–4234. doi:[10.2527/jas.2013-6412](https://doi.org/10.2527/jas.2013-6412)
- Qiu, K., C. F. Qin, M. Luo, X. Zhang, W. J. Sun, N. Jiao, D. F. Li, and J. D. Yin. 2016. Protein restriction with amino acid-balanced diets shrinks circulating pool size of amino acid by decreasing expression of specific transporters in the small intestine. *PLoS One*. 11:e0162475. doi:[10.1371/journal.pone.0162475](https://doi.org/10.1371/journal.pone.0162475)
- Rérat, A., C. Simoes-Nunes, F. Mendy, P. Vaissade, and P. Vaugelade. 1992. Splanchnic fluxes of amino acids after duodenal infusion of carbohydrate solutions containing free amino acids or oligopeptides in the non-anaesthetized pig. *Br. J. Nutr.* 68:111–138. doi:[10.1079/bjn19920071](https://doi.org/10.1079/bjn19920071)
- Roig-Pérez, S., M. Moreto, and R. Ferrer. 2005. Transepithelial taurine transport in caco-2 cell monolayers. *J. Membr. Biol.* 204:85–92. doi:[10.1007/s00232-005-0750-y](https://doi.org/10.1007/s00232-005-0750-y)
- Schutte, J. B., E. J. Van Weerden, and H. L. Bertram. 1983. Sulphur amino acid requirement of laying hens and the effects of excess dietary methionine on laying performance. *Br. Poult. Sci.* 24:319–326. doi:[10.1080/00071668308416746](https://doi.org/10.1080/00071668308416746)
- Siebert, W., H. Ahmadi, and M. Rodehutschord. 2015. Meta-analysis of the influence of dietary glycine and serine, with consideration of methionine and cysteine, on growth and feed conversion of broilers. *Poult. Sci.* 94:1853–1863. doi:[10.3382/ps/pev129](https://doi.org/10.3382/ps/pev129)
- Spangler, H., P. Utterback, C. M. Parsons, and P. Tillman. 2019. Determining the digestible lysine requirement of 22- to 47-week-old Lohmann laying hens using an increasing protein titration methodology. *Poult. Sci.* 98:1706–1715. doi:[10.3382/ps/pey503](https://doi.org/10.3382/ps/pey503)
- Stein, E. D., S. D. Chang, and J. M. Diamond. 1987. Comparison of different dietary amino acids as inducers of intestinal amino acid transport. *Am. J. Physiol.* 252(5 Pt 1):G626–G635. doi:[10.1152/ajpgi.1987.252.5.G626](https://doi.org/10.1152/ajpgi.1987.252.5.G626)
- Takagi, T., R. Ando, A. Ohgushi, T. Yamashita, E. Dobashi, H. Hussain-Yusuf, R. Onodera, T. Bungo, H. Sato, and M. Furuse. 2001. Intracerebroventricular injection of pipelicolic acid inhibits food intake and induces sleeping-like behaviors in the neonatal chick. *Neurosci. Lett.* 310:97–100. doi:[10.1016/S0304-3940\(01\)02059-6](https://doi.org/10.1016/S0304-3940(01)02059-6)
- Tan, B., Y. Yin, Z. Liu, X. Li, H. Xu, X. Kong, R. Huang, W. Tang, I. Shinzato, S. B. Smith, et al. 2009. Dietary L-arginine supplementation increases muscle gain and reduces body fat mass in growing-finishing pigs. *Amino Acids* 37:169–175. doi:[10.1007/s00726-008-0148-0](https://doi.org/10.1007/s00726-008-0148-0)
- Torki, M., A. Mohebbifar, H. A. Ghasemi, and A. Zardast. 2015. Response of laying hens to feeding low-protein amino acid-supplemented diets under high ambient temperature: performance, egg quality, leukocyte profile, blood lipids, and excreta ph. *Int. J. Biomet.* 59:575–584. doi:[10.1007/s00484-014-0870-0](https://doi.org/10.1007/s00484-014-0870-0)
- Vazquez-Anon, M., R. Gonzalez-Esquerria, E. Saleh, T. Hampton, S. Ritcher, J. Firman, and C. D. Knight. 2006. Evidence for 2-hydroxy-4(methylthio) butanoic acid and DL-methionine having different dose responses in growing broilers. *Poult. Sci.* 85:1409–1420. doi:[10.1093/ps/85.8.1409](https://doi.org/10.1093/ps/85.8.1409)
- Wan, J., X. Ding, J. Wang, S. Bai, H. Peng, Y. Luo, Z. Su, Y. Xuan, and K. Zhang. 2017. Dietary methionine source and level affect hepatic sulfur amino acid metabolism of broiler breeder hens. *Anim. Sci. J.* 88:2016–2024. doi:[10.1111/asj.12882](https://doi.org/10.1111/asj.12882)
- Wang, W. W., S. Y. Qiao, and D. F. Li. 2009. Amino acids and gut function. *Amino Acids* 37:105–110. doi:[10.1007/s00726-008-0152-4](https://doi.org/10.1007/s00726-008-0152-4)
- Wolfram, S., H. Giering, and E. Scharer. 1984. Na⁺-gradient dependence of basic amino acid transport into rat intestinal brush border membrane vesicles. *Comp. Biochem. Physiol. A. Comp. Physiol.* 78:475–480. doi:[10.1016/0300-9629\(84\)90581-4](https://doi.org/10.1016/0300-9629(84)90581-4)

- Wright, M. D., and S. C. Loerch. 1988. Effects of rumen-protected amino acids on ruminant nitrogen balance, plasma amino acid concentrations and performance. *J. Anim. Sci.* **66**:2014–2027. doi:[10.2527/jas1988.6682014x](https://doi.org/10.2527/jas1988.6682014x)
- Wu, G. Y. 2009. Amino acids: metabolism, functions, and nutrition. *Amino Acids*. **37**:1–17. doi:[10.1007/s00726-009-0269-0](https://doi.org/10.1007/s00726-009-0269-0)
- Xie, M., S. S. Hou, W. Huang, L. Zhao, J. Y. Yu, W. Y. Li, and Y. Y. Wu. 2004. Interrelationship between methionine and cystine of early Peking ducklings. *Poult. Sci.* **83**:1703–1708. doi:[10.1093/ps/83.10.1703](https://doi.org/10.1093/ps/83.10.1703)
- Yang, W. R., H. Sun, Q. Y. Wang, F. X. Liu, and Z. B. Yang. 2010. Effects of rumen-protected methionine on dairy performance and amino acid metabolism in lactating cows. *Am. J. Anim. Vet. Sci.* **5**:1–7. doi:[10.3844/ajavsp.2010.1.7](https://doi.org/10.3844/ajavsp.2010.1.7)
- Yao, K., Y. Yin, X. Li, P. Xi, J. Wang, J. Lei, Y. Hou, and G. Wu. 2012. Alpha-ketoglutarate inhibits glutamine degradation and enhances protein synthesis in intestinal porcine epithelial cells. *Amino Acids* **42**:2491–2500. doi:[10.1007/s00726-011-1060-6](https://doi.org/10.1007/s00726-011-1060-6)
- Yen, J. T., B. J. Kerr, R. A. Easter, and A. M. Parkhurst. 2004. Difference in rates of net portal absorption between crystalline and protein-bound lysine and threonine in growing pigs fed once daily. *J. Anim. Sci.* **82**:1079–1090. doi:[10.2527/2004.8241079x](https://doi.org/10.2527/2004.8241079x)
- Yin, J., W. Ren, J. Duan, L. Wu, S. Chen, T. Li, Y. Yin, and G. Wu. 2014. Dietary arginine supplementation enhances intestinal expression of SLC7A7 and SLC7A1 and ameliorates growth depression in mycotoxin-challenged pigs. *Amino Acids* **46**:883–892. doi:[10.1007/s00726-013-1643-5](https://doi.org/10.1007/s00726-013-1643-5)