Creatine nitrate supplementation strengthens energy status and delays glycolysis of broiler muscle via inhibition of LKB1/AMPK pathway

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ABSTRACT This study aimed to evaluate the effects of dietary creatine nitrate (**CrN**) on growth performance, meat quality, energy status, glycolysis, and related gene expression of liver kinase B1/AMP-activated protein kinase (**LKB1/AMPK**) pathway in *Pectoralis major* (**PM**) muscle of broilers. A total of 240 male Arbor Acres broilers (28-day-old) were randomly allocated to one of 5 dietary treatments: the basal diet (control group), and the basal diets supplemented with 600 mg/kg guanidinoacetic acid (**GAA**), 300, 600, or 900 mg/kg CrN (identified as GAA_{600}, CrN_{300}, CrN_{600}, or CrN_{900}, respectively). We found that dietary GAA and CrN supplementation for 14 d from d 28 to 42 did not affect broiler growth performance, carcass traits, and textural characteristics of breast muscle. GAA_{600}, CrN_{600}, and CrN_{900} treatments increased pH_{24h} and decreased drip loss of PM muscle compared with the control ($P < 0.05$). The PM muscles of CrN_{600} and CrN_{900} groups showed higher glycogen concentration and lower lactic acid concentration accompanied by lower activities of phosphofructokinase (**PFK**), pyruvate kinase (**PK**), and lactate dehydrogenase (**LDH**) ($P < 0.05$). Simultaneously, GAA_{600} and all CrN treatments increased concentration of muscle creatine, phosphocreatine (**PCr**) and ATP, and decreased AMP concentration and AMP/ATP ratio ($P < 0.05$). Meanwhile, the concentrations of muscle creatine, PCr, and ATP were increased linearly, while muscle AMP concentration and AMP/ATP ratio were decreased linearly and quadratic as the dose of CrN increased ($P < 0.05$). GAA_{600}, CrN_{600}, and CrN_{900} treatments upregulated mRNA expression of *CreaT* in PM muscle, and CrN_{600} and CrN_{900} treatments downregulated *GAMT* expression in liver and PM muscle compared with the control or GAA_{600} groups ($P < 0.05$). The mRNA expression of muscle *LKB1, AMPKα1*, and *AMPKα2* was downregulated linearly in response to the increasing CrN level ($P < 0.05$). Overall, CrN showed better efficacy on strengthening muscle energy status and improve meat quality than GAA at the some dose. These results indicate that CrN may be a potential replacement for GAA as a new creatine supplement.

Key words: broiler, creatine nitrate, meat quality, energy status, glycolysis

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INTRODUCTION

The demand for poultry meat has increased significantly in recent decades as a result of its low cost, more comprehensive nutrition, and convenient for further processing (Petracci et al., 2015). More importantly, an increasing number of consumers favor safe and high-quality poultry meat, which has become the focus of modern poultry industry (Kuźniacka et al., 2020). Previous research has established that age, breed-type, pre-slaughter stress, and rearing environment were the important factors influencing the meat quality of broilers (Bogosavljević-Bošković et al., 2012). Furthermore, diet is also one of the most important key factors affecting meat quality. Dietary supplementation with some exogenous amino acid derivatives, such as creatine monohydrate (**CMH**) and guanidinoacetic acid (**GAA**) have been suggested as an effective way to improve meat quality of broilers (Michiels et al., 2012; Zhang et al., 2017, 2019).

Creatine, also known as N-methyl guanidinoacetic acid, is a natural amino acid derivative. The endogenous synthesis of creatine can be divided into 2 steps: the first step occurs in the kidney and pancreas, where arginine and glycine synthesize GAA under the action of

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L-arginine-glycine amidinotransferase (**AGAT**); and the second step occurs in the liver, where GAA is methylated to creatine by guanidinoacetate methyltransferase (**GAMT**) (Brosnan et al., 2011; Longo et al., 2011). Therefore, GAA is a natural biosynthetic precursor of creatine and can be used as creatine source. Skeletal muscle cells accumulate creatine from the plasma via specific Na^+-dependent creatine transporter (**CreaT**), and then creatine is phosphorylated by creatine kinase to produce phosphocreatine (**PCr**) for the temporary storage of energy (Wyss and Kaddurah-Daouk, 2000). When muscle ATP are overexpend, PCr can provide high-energy phosphate bond to ADP to regenerate ATP (Curt et al., 2015). Therefore, the creatine/PCr system serves as a rapidly mobilizable reserve of high-energy phosphate in skeletal muscle or brain cells that have high and variable energy demands to recycle ATP and maintain energy stability to a certain extent (Michiels et al., 2012; Zhang et al., 2017; Portocarero and Braun, 2021).

Postmortem muscle glycolysis is highly related to meat quality traits of broiler chickens. After being slaughtered, rapid anaerobic glycolysis and over accumulation of H^+ and lactic acid in muscle always accompanied with meat quality deteriorating of broilers, especially in birds subjected to pre-slaughter stress (Hamilton et al., 2003; Zhang et al., 2014; Wang et al., 2017). AMP-activated protein kinase is a metabolic sensor that can sense changes in intracellular energy and regulate energy balance. Prior studies have noted that AMPK plays a significant role in regulating postmortem muscle glycolysis of animals (Shen et al., 2006; Xing et al., 2016; Zhang et al., 2017). Dietary supplementation with creatine additive CMH or its precursor GAA can improve the muscle reserves of creatine, PCr, and ATP, delay the occurrence of rapid muscle glycolysis and the accumulation of muscle lactic acid after slaughter, which help to improve meat quality of broilers (Nissen and Young, 2006; Zhang et al., 2014, 2019). CMH has been identified as the most common form of creatine supplements in the field of human sports medicine (Kreider et al., 2017). However, CMH has some limitations, such as its low solubility in water and the oral bioavailability of CMH was less than complete (Alraddadi et al., 2018).

As a precursor of creatine, GAA has been widely used to promote growth performance and muscle development, increase energy reserve and improve meat quality of broilers (Córdova-Noboa et al., 2018; DeGroot et al., 2019). The European Union authorized GAA as a nutritional additive for improving the performance at dosage of 600 to 1,200 mg/kg in chicken diets. Many previous studies have found that addition of 600 mg/kg GAA to broiler diet can strength the reserve of breast muscle energy substances by increasing the concentrations of creatine and PCr as well as the ATP/AMP ratio (Michiels et al., 2012; Zhang et al., 2019; Zarghi et al., 2020), increase breast meat yield and reduce the severity of wooden breast myopathy (Córdova-Noboa et al., 2018). However, exogenous GAA requires more methyl supply to synthesize creatine, which may limit the methyl required for other transmethylation reactions (Ibrahim et al., 2019). Creatine nitrate (**CrN**) is a novel form of creatine, which has greater solubility and muscle retention (Galvan et al., 2016). Moreover, compared with CMH and GAA, CrN has a lower cost. We, thus, hypothesized that dietary CrN has good potential as a substitute for GAA in poultry industry. To date, although the safety and efficacy of CrN to human has been verified (Joy et al., 2014; Dalton et al., 2017), little is known about of the effects of CrN on meat quality and muscle energy metabolism of broilers. Therefore, this study aims to evaluate the possibility effects of CrN as a substitute for GAA by investigating muscle energy status, glycolysis metabolism, and meat quality of broiler chickens fed diets supplementation with graded levels of CrN.

MATERIALS AND METHODS

Birds, Diets, and Experimental Design

All experimental procedures obtained ethical approval by the Institutional Animal Care and Use Committee of Nanjing Agricultural University. A total of 280 Arbor Acres male broiler chickens (28-day-old) with similar body weight (mean ± SD, 1,440.88 ± 4.40 g) were randomly allocated to one of 5 dietary treatments, with 6 replicate cages and 8 birds per cage (120 cm × 80 cm × 45 cm). These 5 dietary treatments included: the basal diet (control group), and the basal diet supplemented with 600 mg/kg GAA (identified as GAA_{600}), 300, 600, or 900 mg/kg of CrN (identified as CrN_{300}, CrN_{600}, or CrN_{900}, respectively). The experiment lasted 14 d from 28 to 42 d. Both GAA and CrN (purity ≥99%) were purchased from Tianjin Tiancheng Pharmaceutical Co., Ltd. (Tianjin, China). All birds were raised in three-tier wired cages and allowed access to feed and water in a temperature-controlled room maintained at 22°C during the trial period from 28 to 42 d. The basal diet formulation and nutritional levels are presented in Table 1. The diets were fed in pellet form. All birds were weighed at 28 and 42 d of age to calculate the average daily feed intake (**ADFI**), average daily gain (**ADG**), and feed conversion ratio (**FCR**; feed: gain, g:g).

Slaughter and Sample Collection

On 42 d of age, 2 birds close to the average BW per replicate (cage) were selected. After electrically stunning in a salt-water bath (1% NaCl, wt/vol) with a constant voltage 50 V at 400 Hz for 5 s each bird, the chickens were then immediately slaughtered via exsanguination. Carcass weight was measured after defeathering. The head, neck, and feet were removed, and then the carcasses were eviscerated and weighted to determine the percentage of eviscerated yield. Dressing percentage was calculated by dividing the carcass weight by live body weight (**BW**). Abdominal fat (leaf fat surrounding the

Table 1. Basal diet formulation and nutritional values.

	Grower stage (22−42 d)
Ingredient (%)	
Corn	59.37
Soybean meal	31.90
Soybean oil	5.00
Limestone	1.23
Dicalcium phosphate	1.50
L-Lysine·HCl	0.11
DL-Methionine	0.27
Salt	0.30
Vitamin premix [1]	0.03
Mineral premix [2]	0.20
70% Choline chloride	0.09
Nutrient level (calculated, %)	
Metabolisable energy (MJ/kg)	12.97
Crude protein	19.00
Calcium	0.90
Total phosphorus	0.56
Available phosphorus	0.35
Lysine	1.00
Methionine	0.46
Methionine + cystine	0.80

[1]Vitamin premix provided per kilogram of diet: vitamin A, 12,000 IU; vitamin D_3, 2,500 IU; vitamin E, 11 mg; menadione, 1.3 mg; thiamine, 2.21 mg; riboflavin, 7.8 mg; nicotinamide, 40 mg; calcium pantothenate, 16.5 mg; pyidoxine·HCl, 4 mg; biotin, 0.04 mg; folic acid, 1.2 mg; vitamin B_{12}, 15 μg.

[2]Mineral premix provided per kilogram of diet: iron, 80 mg; copper, 8 mg; manganese, 110 mg; zinc, 65 mg; iodine, 1.1 mg; selenium, 0.3 mg.

cloaca and abdominal fat surrounding the gizzard) and the breast and thigh muscle were removed and weighed to calculate their relative yield to eviscerated weight. Within 15 min, 2.0 g of liver and 3.0 g of muscle samples from the right *Pectoralis major* (**PM**) were put into RNase-free tables, snap-frozen in liquid nitrogen and stored at −80°C for further analysis. The entire left PM muscles were collected and stored at 4°C for meat quality measurements and texture profile analyses.

Meat Quality Measurements

The pH values of breast muscle at 45 min and 24 h postmortem were performed using a FiveGo pH Meter F2 (Mettler-Toledo AG, Analytical., Shanghai, China). The pH probe was inserted at an angle of 45° into the muscle directly and took the average value of 3 measurements as the final result (Zhang et al., 2014). At 24 h postmortem, the color parameters, including L* (lightness), a* (redness), and b* (yellowness) values, were measured by using a CR410 chroma meter (Konica Minolta Sensing Inc., Osaka, Japan). Each sample was measured 3 times at different positions, and the average value is taken as the final result. The drip loss, cooking loss, and shear force value of PM muscle at 24 h postmortem were determined as described previously (Zhang et al., 2014).

Texture Profile Analyses

The textural characteristics, including hardness (N), cohesiveness, springiness (mm), gumminess (N), and chewiness (mJ), were assessed by using a texture profile analyzer (**TPA**) (TMS-Pro, FTC, Sterling, VA) following the method according to Gurikar et al. (2014). Each cooked sample was cooled to room temperature and then cut into a cylinder with a diameter of 2 cm and a height of 1 cm by using a special sampler, which was placed on the sample determination platform of texture analyzer. The test settings of the texture analyzer were as follows: probe model P/50, pretest speed 2.0 mm/s, test speed 1.0 mm/s, post-test speed 1.0 mm/s, compression ratio 40%, time between two presses 5 s and load type auto −5 g.

Muscle Lactic Acid, Glycogen, and Glycolytic Potential Determination

The glycogen concentration in frozen muscle sample was determined as previously described (Zhang et al., 2014). The concentration of muscle lactic acid was measured by using a commercial diagnoses kit (Nanjing Jiancheng Biochemical Institute, Nanjing, China). Glycolytic potential (**GP**) was calculated according to the following formula: GP = 2 × [glycogen] + [lactic acid], and the result was expressed as μmol/g of lactic acid equivalent in wet muscle (Monin and Sellier, 1985).

Activity Analysis of Muscle Glycolytic Key Enzymes

Frozen muscle sample of exactly 0.50 g was homogenized in centrifuge tube containing 4.5 mL ice-cold physiological saline (0.75%) solution and centrifuged at 3,500 × *g* for 10 min at 4°C. The enzymes activities of hexokinase (**HK**), phosphofructokinase (**PFK**), pyruvate kinase (**PK**), and lactate dehydrogenase (**LDH**) in the supernatant were measured spectrophotometrically with commercial diagnose kits (Nanjing Jiancheng Biochemical Institute).

Muscle Creatine, Phosphocreatine, and Adenosine Phosphate Determination

The concentrations of muscle creatine, PCr, ATP, ADP, and AMP were determined by HPLC method as previous described (Zhang et al., 2017). The frozen muscle samples were extracted by 5% perchloric acid for creatine and PCr extraction, and 7% perchloric acid for ATP, ADP, and AMP extraction. The supernatant used for creatine and PCr analysis was adjusted to a pH of 7.0 with 0.8 *M* K_2CO_3, and the supernatant used for adenosine phosphate analysis was adjusted to a pH of 6.5 with 1.03 *M* KOH. The final supernatant was filtered with a 0.45 μm membrane, and then 10 μL (for the determination of ATP, ADP, and AMP) or 20 μL (for the determination of creatine and PCr) of the relative sample solution was separated on an UltiMate 3000 HPLC system (Thermo Fisher Scientific, San Jose, CA) equipped with a Waters SunFire C18 column (250 mm × 4.6 mm id, 5 μm) at 25°C for creatine and PCr determination, and at 30°C for ATP, ADP and AMP

determination. The mobile phase was a mixture of methyl cyanides and 29.4 mM KH_2PO_4 buffer (volume ratio = 2:98) for creatine and PCr determination and was a mixture of methanol and phosphate buffer (volume ratio = 13.5:86.5) for ATP, ADP and AMP determination. The flow rate was 1.0 mL/min. The standard samples of creatine-disodium salt, PCr-disodium salt, 5'-ATP-disodium salt, 5'-ADP sodium salt, and 5'-AMP sodium salt (Sigma-Aldrich, St. Louis, MO) were used to establish standard curves.

Total RNA Extraction and Real-Time Quantitative PCR Analysis

Total RNA from the frozen muscle sample was extracted using the RNAiso Plus reagent (Takara Biotechnology Co. Ltd., Dalian, China). Then, the concentration, quantity and quality of total RNA were tested with a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Inc., Wilmington, DE). Total RNA with the OD_{260}/OD_{280} ratio at 1.8 to 2.0 was used for subsequent PCR reactions. The purified total RNA was reverse transcribed into cDNA with the primescript RT Master Mix kit (Takara Biotechnology Co. Ltd.), and the synthesized cDNA products were stored at −20°C until use. Real-time quantitative PCR (**RT-qPCR**) analyses were performed in the CFX ConnectTM Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA) using SYBR Premix Ex Taq kits (Takara Biotechnology Co. Ltd.). The specific primer sequences used in the present study are listed in Table 2. The mRNA expression level of housekeeping gene (β-actin) was used to normalize the cycle threshold (**Ct**) values. The expression level of target genes relative to β-actin were calculated using the $2^{-\Delta\Delta Ct}$ method and expressed as the relative fold change to the control group (Livak and Schmittgen, 2001).

Statistical Analysis

Data were analyzed with the use of SPSS statistical software (Version 20.0 for windows, SPSS Inc., Chicago, IL) for one-way analysis of variance (**ANOVA**). The observed indexes were analyzed by the mean of 2 chickens per cage as a replicate except ADFI, ADG, and FCR were analyzed by the cage as a replicate (n = 6). Besides, orthogonal contrasts were used to examine the linear and quadratic effects in response to increasing the dietary supplementation of CrN among the control, CrN_{300}, CrN_{600}, and CrN_{900} groups. The results are presented as mean values and the standard error of the mean (**SEM**). A probability level of $P < 0.05$ was considered to indicate statistical significance.

RESULTS

Growth Performance

As exhibited in Table 3, no significant treatment differences were observed in ADFI, ADG, and FCR of birds during the trial period from 28 to 42 d ($P > 0.05$).

Carcass Traits, Meat Quality, and Textural Characteristics

In the present study, although dietary supplementation with 600 mg/kg of GAA, and 300, 600, or 900 mg/kg of CrN from 28 to 42 d had no significant effect on carcass traits of birds slaughtered at 42 d of market age ($P > 0.05$; Table 4). The thigh muscle yield was increased linearly in response to the increase in dietary CrN level ($P < 0.05$).

As shown in Table 5, the breast muscle of GAA_{600}, CrN_{600}, and CrN_{900} groups showed higher pH_{24h} than were seen in the control group ($P < 0.05$). The pH_{24h} was increased linearly in response to the increasing CrN supplementation level ($P < 0.01$). Moreover, compared with the control group, the drip loss of breast muscle in GAA_{600} and all CrN groups showed a significant reduction ($P < 0.05$). As the dose of CrN increased, the drip loss of breast muscle was decreased in linear and quadratic manners ($P < 0.05$). Meanwhile, chewiness and

Table 2. Primer specific sequences used for RT-qPCR analysis.

Genes	Primer sequence (5′-3′)	Amplicon size (bp)	Genbank identification
GAMT	F: CGTGAAGGGCAAATACAGCG R: GGAAGGAGTAGTAGCGGCAC	146	XM_040692773.1
CreaT	F: TGAACTACAAACCGCTGACG R: GCTCGTAGATAACGGTGCAG	120	JN628439.2
LKB1	F: TGAGAGGGATGCTTGAATACGA R: ACTTGTCCTTTGTTTCTGGGC	138	NM_001045833.1
MO25α	F: CGTGTTTAAGGTGTTTGTAGCC R: AGCAACTGCTGAATTTGGGT	245	XM_015277058.3
STRADα	F: TAAACCCGAACGGATTAGGCG R: TGCTGTCTGGGAGGAAGTTG	182	NM_001305191.1
AMPKα1	F: ATCTGTCTCGCCCTCATCCT R: CCACTTCGCTCTTCTTACACCTT	125	NM_001039603.1
AMPKα2	F: GGGACCTGAAACCAGAGAACG R: ACAGAGGAGGGCATAGAGGATG	215	NM_001039605.1
β-actin	F: ATCCGGACCCTCCATTGTC R: AGCCATGCCAATCTCGTCTT	120	NM_205518.1

Abbreviations: *AMPKα1*, adenosine 5′-monophosphate-activated protein kinase α1; *AMPKα2*, adenosine 5′-monophosphate-activated protein kinase α2; *CreaT*, creatine transporter; *GAMT*, S-adenosyl-L-methionine: guanidinoacetate N-methyltransferase; *LKB1*, liver kinase B1; *MO25α*, mouse protein 25α; *STRADα*, STE20-related adaptor α.

Table 3. Effects of dietary graded creatine nitrate (CrN) supplementation on growth performance of broilers from 28 to 42 d of age.

Items	Treatments[1]					SEM	*P* value		
	Control	GAA_{600}	CrN_{300}	CrN_{600}	CrN_{900}		ANOVA	Linear[2]	Quadratic[2]
Initial BW at 28 d (g/bird)	1,438.00	1,442.50	1,440.00	1,441.50	1,441.88	0.80	0.422	-	-
BW at 42 d (g/bird)	2,798.50	2,826.57	2,839.50	2,841.86	2,856.04	15.60	0.840	0.300	0.719
ADFI (g/ day·bird)	169.24	174.58	175.82	176.62	175.41	1.76	0.722	0.311	0.359
ADG (g/ day·bird)	97.18	98.86	99.97	100.03	101.01	1.12	0.869	0.339	0.737
FCR (feed:gain, g:g)	1.74	1.77	1.76	1.77	1.74	0.01	0.649	0.925	0.222

The data are represented as the mean value and pooled SEM (n = 6).

Abbreviations: ADFI, average daily feed intake; ADG, average daily gain; FCR, feed conversion ratio.

[1]Control, basal diet; GAA_{600}, basal diet supplemented with 600 mg/kg guanidinoacetic acid (GAA); CrN_{300}, CrN_{600}, and CrN_{900}, basal diets supplemented with 300, 600, and 900 mg/kg CrN, respectively.

[2]Orthogonal polynomials were used to estimate the linear and quadratic effects of dietary CrN supplementation among the control, CrN_{300}, CrN_{600}, and CrN_{900} groups.

Table 4. Effects of dietary graded creatine nitrate (CrN) supplementation on carcass traits of broilers.

Items	Treatments[1]					SEM	*P* value		
	Control	GAA_{600}	CrN_{300}	CrN_{600}	CrN_{900}		ANOVA	Linear[2]	Quadratic[2]
Dressing percentage (%)	95.19	94.65	95.52	95.05	94.98	0.11	0.183	0.196	0.288
Eviscerated yield (%)	78.56	78.29	79.81	79.91	79.77	0.24	0.082	0.054	0.108
Breast muscle yield (%)	25.32	25.65	25.37	26.09	26.15	0.27	0.801	0.101	0.977
Thigh muscle yield (%)	17.93	19.10	18.29	18.83	18.90	0.20	0.346	0.037	0.691
Abdominal fat percentage %)	1.71	1.53	1.69	1.59	1.52	0.05	0.619	0.128	0.807

The data are represented as the mean value and pooled SEM (n = 6).

[1]Control, basal diet; GAA_{600}, basal diet supplemented with 600 mg/kg guanidinoacetic acid (GAA); CrN_{300}, CrN_{600}, and CrN_{900}, basal diets supplemented with 300, 600, and 900 mg/kg CrN, respectively.

[2]Orthogonal polynomials were used to estimate the linear and quadratic effects of dietary CrN supplementation among the control, CrN_{300}, CrN_{600}, and CrN_{900} groups.

gumminess of the PM muscle were decreased linearly in response to the increase in dietary CrN level ($P < 0.05$).

Concentrations of Muscle Glycogen, Lactic Acid, GP, and Activities of Muscle Glycolytic Enzymes

Both CrN_{600} and CrN_{900} treatments increased the concentrations of muscle glycogen and reduced the concentrations of lactic acid as compared with the control and CrN_{300} groups ($P < 0.05$; Table 6). The enzyme activities of PFK were lower in muscles of CrN_{600} and CrN_{900} treatments than were seen in the control, GAA_{600} and CrN_{300} treatments ($P < 0.05$). Moreover, the activities of muscle PK were lower in GAA_{600} and all CrN treatments, and the activities of muscle LDH were lower in all CrN supplementation groups than those were seen in the control group ($P < 0.05$). The muscle

Table 5. Effects of dietary graded creatine nitrate (CrN) supplementation on meat quality and textural characteristics of the *Pectoralis major* muscle of broilers.

Items	Treatments[1]					SEM	*P* value		
	Control	GAA_{600}	CrN_{300}	CrN_{600}	CrN_{900}		ANOVA	Linear[2]	Quadratic[2]
Meat quality									
pH_{45min}	6.17	6.20	6.23	6.19	6.22	0.01	0.630	0.427	0.561
pH_{24h}	5.86[b]	5.92[a]	5.90[ab]	5.93[a]	5.95[a]	0.01	0.015	<0.001	0.556
L* (lightness)	42.54	42.58	42.78	42.72	44.37	0.28	0.217	0.086	0.252
a* (redness)	1.41	1.37	1.29	1.30	1.33	0.07	0.976	0.738	0.629
b* (yellowness)	3.13	3.27	3.72	3.77	3.67	0.12	0.291	0.118	0.147
Drip loss (%)	2.69[a]	2.25[b]	2.26[b]	2.23[b]	2.36[b]	0.05	0.006	0.018	0.004
Cooking loss (%)	13.07	12.43	12.05	11.31	11.37	0.33	0.426	0.086	0.469
Shear force (N)	20.15	20.14	21.32	21.71	22.33	0.52	0.630	0.193	0.819
Textural characteristics									
Hardness (N)	22.26	21.44	21.28	20.40	20.84	0.62	0.923	0.357	0.571
Cohesiveness (-)	0.46	0.43	0.45	0.43	0.43	0.01	0.676	0.174	0.751
Springiness (mm)	2.85	2.88	2.74	2.90	2.65	0.05	0.488	0.301	0.676
Gumminess (N)	11.08	10.18	10.63	9.11	9.61	0.33	0.388	0.047	0.468
Chewiness (mJ)	31.47	28.06	29.94	24.68	24.87	0.90	0.057	0.001	0.594

Abbreviations: $pH_{45\ min}$, pH at 45 min postmortem; pH_{24h}, pH at 24 h postmortem.

[a,b]Different letters in the mean value of the same row indicate a significant difference ($P < 0.05$). The data are represented as the mean value and pooled SEM (n = 6).

[1]Control, basal diet; GAA_{600}, basal diet supplemented with 600 mg/kg guanidinoacetic acid (GAA); CrN_{300}, CrN_{600}, and CrN_{900}, basal diets supplemented with 300, 600, and 900 mg/kg creatine nitrate (CrN), respectively.

[2]Orthogonal polynomials were used to estimate the linear and quadratic effects of dietary CrN supplementation among the control, CrN_{300}, CrN_{600}, and CrN_{900} groups.

Table 6. Effects of dietary graded creatine nitrate (CrN) supplementation on glycolytic parameters and activities of glycolytic enzymes in *Pectoralis major* muscle of broilers.

	Treatments[1]						*P* value		
Items	Control	GAA_{600}	CrN_{300}	CrN_{600}	CrN_{900}	SEM	ANOVA	Linear[2]	Quadratic[2]
Glycolytic parameters									
Glycogen (μmol/g)	5.26[b]	5.54[b]	5.15[b]	6.34[a]	6.53[a]	0.14	0.001	<0.001	0.513
Lactic acid (μmol/g)	131.42[a]	126.34[ab]	130.79[a]	125.43[b]	125.56[b]	0.85	0.042	0.010	0.844
GP (μmol/g)	141.94	137.42	141.08	138.11	138.62	0.81	0.321	0.132	0.717
Activities of glycolytic enzymes									
HK (U/mg of protein)	14.44	12.34	13.88	12.51	11.67	0.69	0.722	0.208	0.936
PFK (U/mg of protein)	56.93[a]	53.59[a]	52.80[a]	47.61[b]	45.66[b]	0.92	<0.001	<0.001	0.480
PK (U/g of protein)	51.76[a]	38.92[b]	36.18[b]	34.99[b]	38.93[b]	1.48	0.001	0.002	0.001
LDH (U/mg of protein)	4.30[a]	3.61[ab]	3.08[b]	3.15[b]	2.97[b]	0.13	0.003	0.001	0.044

Abbreviations: GP, glycolytic potential (GP = 2 × [glycogen] + [lactic acid]); HK, hexokinase; LDH, lactate dehydrogenase; PFK, phosphofructokinase; PK, pyruvate kinase.

[a,b]Different letters in the mean value of the same row indicate a significant difference ($P < 0.05$). The data are represented as the mean value and pooled SEM (n = 6).

[1]Control, basal diet; GAA_{600}, basal diet supplemented with 600 mg/kg guanidinoacetic acid (GAA); CrN_{300}, CrN_{600}, and CrN_{900}, basal diets supplemented with 300, 600, and 900 mg/kg CrN, respectively.

[2]Orthogonal polynomials were used to estimate the linear and quadratic effects of dietary CrN supplementation among the control, CrN_{300}, CrN_{600}, and CrN_{900} groups.

glycogen concentration was increased linearly ($P < 0.01$), and the concentration of lactic acid and activity of PFK were decreased linearly ($P < 0.01$) in response to the increasing CrN supplementation level. Meanwhile, the activities of PK and LDH were decreased in linear ($P < 0.01$) and quadratic ($P < 0.05$) manners as the dose of CrN increased.

Concentrations of Muscle Creatine, PCr, ATP, ADP, and AMP

As shown in Table 7, GAA_{600} and all CrN treatments increased the concentrations of muscle creatine and ATP ($P < 0.05$), and decreased the concentration of muscle AMP and AMP/ATP ratio ($P < 0.05$). Moreover, the concentrations of PCr were higher in muscles of GAA_{600}, CrN_{600}, and CrN_{900} groups than were seen in the control group ($P < 0.05$). What's more, the muscle creatine concentration in CrN_{900} group was higher than that in the GAA_{600} group ($P < 0.05$). The concentrations of muscle creatine, PCr and ATP were increased linearly in response to the increasing CrN supplementation level ($P < 0.01$). Meanwhile, the muscle AMP concentration and AMP/ATP ratio were decreased linearly ($P < 0.05$) and quadratic ($P < 0.05$) in response to the increasing CrN supplementation level. There was no significant difference in concentrations of muscle PCr, ATP, AMP, and AMP/ATP ratio among GAA_{600} and all CrN treatments ($P > 0.05$).

Relative Gene mRNA Expression of GAMT and CreaT in Liver and PM Muscle

As exhibited in Figure 1, CrN_{600} and CrN_{900} treatments downregulated the liver *GAMT* mRNA expression, and upregulated the muscle *CreaT* mRNA expression compared with the control group ($P < 0.05$). Birds in the GAA_{600} group showed higher the mRNA expression of liver *GAMT* and muscle *CreaT* than those in the control group ($P < 0.05$). Moreover, CrN_{600} and CrN_{900} treatments downregulated the relative mRNA expression of *GAMT* both in liver and PM muscle compared with the GAA_{600} treatment ($P < 0.05$). The mRNA expression levels of *GAMT* both in liver and PM muscle were decreased linearly in response to the increasing CrN supplementation level ($P < 0.05$). Meanwhile, the mRNA expression of muscle *CreaT* was up-

Table 7. Effects of dietary graded creatine nitrate (CrN) supplementation on energy status in *Pectoralis major* muscle of broilers.

	Treatments[1]						*P* value		
Items	Control	GAA_{600}	CrN_{300}	CrN_{600}	CrN_{900}	SEM	ANOVA	Linear[2]	Quadratic[2]
Creatine (μmol/g)	11.99[c]	12.98[b]	12.67[b]	12.61[b]	13.77[a]	0.12	<0.001	<0.001	0.232
PCr (μmol/g)	1.50[b]	2.01[a]	1.72[ab]	1.87[a]	1.99[a]	0.06	0.018	0.001	0.599
PCr/Creatine ratio	0.13	0.15	0.14	0.15	0.14	0.01	0.531	0.102	0.416
ATP (μmol/g)	1.20[b]	1.93[a]	1.99[a]	1.96[a]	1.98[a]	0.09	0.022	0.009	0.046
ADP (μmol/g)	0.74	0.76	0.87	0.79	0.79	0.02	0.361	0.616	0.084
AMP (μmol/g)	0.35[a]	0.23[b]	0.24[b]	0.25[b]	0.26[b]	0.01	0.021	0.036	0.041
AMP/ATP ratio	0.29[a]	0.12[b]	0.12[b]	0.13[b]	0.13[b]	0.01	<0.001	<0.001	<0.001

Abbreviations: ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate; PCr, phosphocreatine.

[a,b,c]Different letters in the mean value of the same row indicate a significant difference ($P < 0.05$). The data are represented as the mean value and pooled SEM (n = 6).

[1]Control, basal diet; GAA_{600}, basal diet supplemented with 600 mg/kg guanidinoacetic acid (GAA); CrN_{300}, CrN_{600}, and CrN_{900}, basal diets supplemented with 300, 600, and 900 mg/kg CrN, respectively.

[2]Orthogonal polynomials were used to estimate the linear and quadratic effects of dietary CrN supplementation among the control, CrN_{300}, CrN_{600}, and CrN_{900} groups.

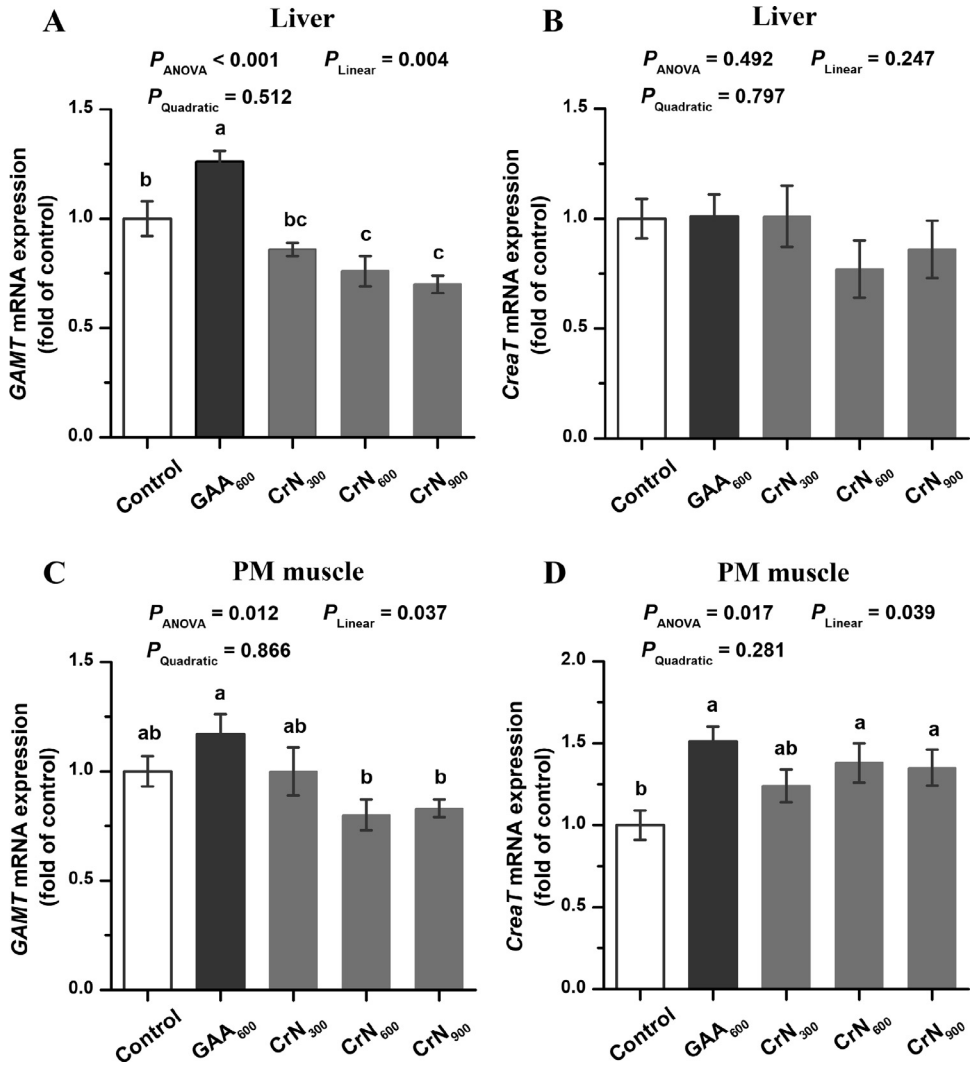

Figure 1. Effects of dietary graded creatine nitrate (CrN) supplementation on the relative mRNA expression for *GAMT* and *CreaT* in the liver and *Pectoralis major* (PM) muscle of broilers. The data are represented as the mean value ± SEM (n = 6). Means without a common letter (a, b or c) significantly differ ($P < 0.05$). Orthogonal polynomials were used to estimate the linear and quadratic effects of dietary CrN supplementation among the control, CrN_{300}, CrN_{600}, and CrN_{900} groups. Abbreviations: *CreaT*, creatine transporter; Control, basal diet; CrN_{300}, CrN_{600}, and CrN_{900}, basal diets supplemented with 300, 600, and 900 mg/kg CrN, respectively; GAA_{600}, basal diet supplemented with 600 mg/kg guanidinoacetic acid (GAA); *GAMT*, S-adenosyl-L-methionine: guanidinoacetate N-methyltransferase.

regulated linearly with increasing dietary CrN level ($P < 0.05$).

Relative Gene mRNA Expression of LKB1/AMPK Pathway in PM Muscle

According to the Figure 2, GAA_{600} and all CrN groups showed lower mRNA expression of *LKB1* and *AMPKα2* in PM muscle than those from the control group ($P < 0.05$). The mRNA expression levels of *LKB1, AMPKα1*, and *AMPKα2* in PM muscle were downregulated linearly in response to the increasing CrN supplementation level ($P < 0.05$). There was no significant difference in the mRNA expression of *MO25α* and *STRADα* in muscle among all experimental treatments ($P > 0.05$).

DISCUSSION

Some previous studies showed that dietary long-term (>35 d) supplementation with GAA at dose rate of 600 to 1,200 mg/kg can improve the feed conversion efficiency and increase body weight gain of broilers (Michiels et al., 2012; Córdova-Noboa et al., 2018; He et al., 2019), and even under stress condition (Majdeddin et al., 2020). Faraj et al. (2014) reported that dietary addition of 4 to 12 g/kg CMH for a trial period of 42 d can improve birds' growth performance. In this study, dietary supplementation with 600 mg/kg of GAA, and 300 to 900 mg/kg of CrN for 14 d prior to slaughter had no significant effect on ADFI, ADG, FCR, and carcass traits of broilers. Similarly, earlier studies have shown that addition 600 and 1,200 mg/kg CMH or GAA to diets for 2 wk prior to slaughter had no significant effect on the growth performance and carcass traits of broilers (Zhang et al., 2014, 2019). These inconsistent results may be due to the differences in the types of creatine supplements, the level of dosage, bioavailability as well as the duration of trials.

Recently, consumers have become more critical toward meat quality when purchasing meat products.

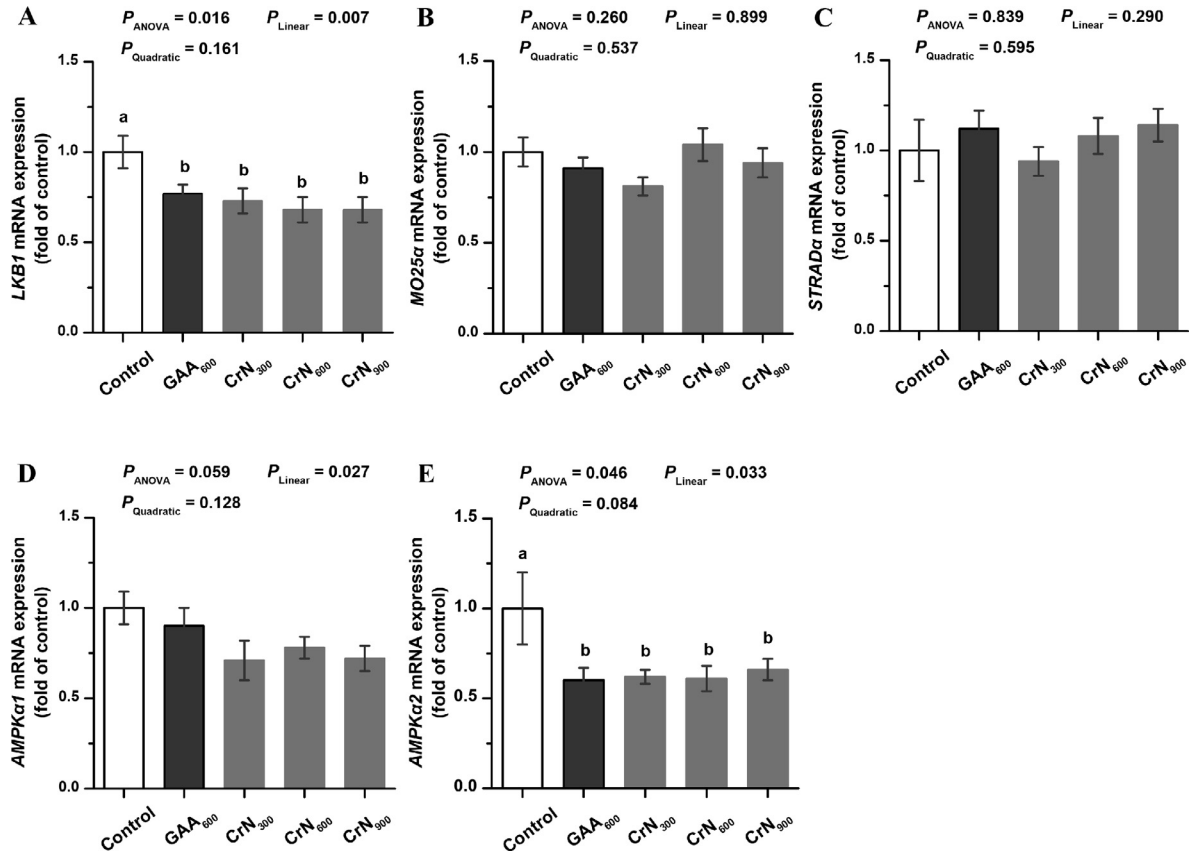

Figure 2. Effects of dietary graded creatine nitrate (CrN) supplementation on the relative mRNA expression of *LKB1, MO25α, STRADα, AMPKα1* and *AMPKα2* in *Pectoralis major* muscle of broilers. The data are represented as the mean value ± SEM (n = 6). Means without a common letter (a or b) significantly differ ($P < 0.05$). Orthogonal polynomials were used to estimate the linear and quadratic effects of dietary CrN supplementation among the control, CrN_{300}, CrN_{600}, and CrN_{900} groups. Abbreviations: *AMPKα1*, adenosine 5′-monophosphate-activated protein kinase α1; *AMPKα2*, adenosine 5′-monophosphate-activated protein kinase α2; Control, basal diet; CrN_{300}, CrN_{600}, and CrN_{900}, basal diets supplemented with 300, 600, and 900 mg/kg CrN, respectively; GAA_{600}, basal diet supplemented with 600 mg/kg guanidinoacetic acid (GAA); *LKB1*, liver kinase B1; *MO25α*, mouse protein 25α; *STRADα*, STE20-related adaptor α.

Generally, meat products with high quality traits are easy to be accepted by consumers. The pH is an important index to reflect meat quality. With the development of postmortem glycolysis, the contents of ATP and glycogen decreased continuously, and lactic acid accumulated continuously, resulting in the decrease of muscle pH, which finally leads to the degeneration of muscle protein (Ryu and Kim, 2005). In the present study, the muscle pH_{24h} of the CrN_{600} and CrN_{900} groups was higher than those of the control and GAA groups, indicating that dietary addition of CrN could attenuate the rapid decline of muscle pH. Water-holding capacity (**WHC**) is also one important attribute reflecting meat quality as it can be directly used to evaluate the ability of fresh muscle maintains its own moisture (Pearce et al., 2011). Generally, inferior WHC can negatively affect the meat color, tenderness, flavor, and nutrients, and thus has important economic significance (Hughes et al., 2014). The results of this study showed that dietary supplementation with 600 mg/kg GAA, or 300, 600, and 900 mg CrN for 2 wk before slaughter significantly enhanced the WHC of breast muscle by reducing drip loss, which is consistent with previous report that addition of GAA or CMH to broiler diets at dose rate of 600 or 1,200 mg/kg enhanced the WHC of breast muscle (Zhang et al., 2017, 2019). During the transformation of muscle to meat, the postmortem glycolysis rate of muscle is closely related to the accumulation of lactate and H^+, which subsequently affects multiple meat quality traits, such as pH, WHC, color, and tenderness (Bee et al., 2007). We thus further analyzed the muscle glycolytic parameters and activities of glycolytic enzymes in the PM muscle of broilers.

After the livestock and poultry are slaughtered, the blood flow stops, and the supply of oxygen and nutrients is cut off. In order to maintain short-term metabolic activities, muscle cells transform from aerobic respiration to anaerobic glycolysis to provide ATP. Glycogen is the main energy reserve of skeletal muscle. GP is an index to measure the content of carbohydrate compounds, including glycogen, glucose, and glucose-6-phosphate that can be converted into lactic acid in muscle (Monin and Sellier, 1985). In this study, higher glycogen concentration and lower lactic acid concentration in breast muscles was accompanied with higher pH_{24h} and lower drip loss in birds of the CrN_{600} and CrN_{900} groups, suggesting that CrN could increase muscle pH and WHC by delaying postmortem glycolysis and reducing the production of lactic acid. More importantly, CrN showed better efficacy than GAA at the some dose. Some key enzymes of anaerobic metabolism mediate the glycolysis metabolic pathway, including HK, PFK, PK, and LDH. These

enzymes catalyze the conversion of glucose to glucose-6-phosphate, glucose-6-phosphate to glucose-1,6-phosphate, phosphoenol pyruvate to pyruvate, and pyruvate to lactic acid under anaerobic condition, respectively (Scheffler and Gerrard, 2007; Nelson and Cox, 2008). High phosphorylation level of PK is conducive to rapid muscle glycolysis and rapid decline of pH, thus affecting meat quality (Huang et al., 2011). In the current study, muscle from CrN_{600} and CrN_{900} groups showed lower activities of PFK than that in the control and GAA_{600}, and CrN_{300} groups. Meanwhile, the activities of muscle PK were lower in GAA_{600} and all CrN groups than those in the control group, and the activities of LDH decreased linearly in response to the increasing CrN supplementation level. These results indicated that dietary addition of CrN reduced the rate of glycolysis reaction and weakened lactic acid accumulation by inhibiting the activities of PFK, PK, and LDH in muscles.

Muscle creatine/PCr system is important for storing and transmitting phosphate-bound energy. When ATP level in muscle is lower than the threshold, PCr transfers its high-energy phosphorylation group to ADP to synthesize ATP again under the action of creatine kinase (Wallimann et al., 2011). ATP is a kind of high-energy phosphate compound, which can be transformed with ADP to store and release energy, thus ensuring the energy supply of various life activities of muscle cells (Cain et al., 1962). In addition, anaerobic glycolysis is also an important bioenergy system involved in the resynthesis of ATP, which provides energy for muscle activity (Wells et al., 2009). Previous studies have demonstrated that addition of 1,200 mg/kg CMH or GAA to the diet increased the concentrations of muscle creatine or PCr of broilers experienced 3-h pre-slaughter transport (Zhang et al., 2017, 2019; Zhang et al., 2021). Herein, we observed that GAA_{600}, and all CrN groups increased the concentrations of muscle creatine and ATP, and reduced muscle AMP content and AMP/ATP ratio compared with control group. In addition, the concentration of muscle PCr in GAA_{600}, CrN_{600}, and CrN_{900} groups were higher than that in the control group. These findings suggest that GAA and CrN supplementation promoted muscle creatine and PCr loading, strengthened the buffer capacity of creatine/PCr pool in muscle, which could quickly afford adequate ATP for skeletal muscle cells to further attenuate glycolytic metabolism. These metabolic changes eventually delayed the muscle glycolysis, reduced the accumulation of lactic acid and the fast reduction of pH, and helped to improve meat quality. Under normal physiological conditions, GAA can be endogenously synthesized from arginine and glycine in kidney and pancreas under the catalysis of AGAT; after GAA transport to the liver, GAMT catalyzes the endogenous synthesis of creatine from S-adenosylmethionine and GAA (Wyss and Kaddurah-Daouk, 2000; Longo et al., 2011). *CreaT* is responsible for transport of endogenously synthesized and ingested creatine to muscle cells, which is the main way for tissues and cells to take up creatine (Wyss and Kaddurah-Daouk, 2000; Brault et al., 2003). In this study, GAA_{600}, CrN_{600}, and CrN_{900} group upregulated the gene expression of *CreaT* in PM muscle in comparison with the control group, suggesting that GAA and CrN supplementation improved the absorption of creatine by skeletal muscle cells via directly activate CreaT on the cell membrane. Zhang et al. (2019) reported that dietary supplementation with 600 and 1,200 mg/kg GAA upregulated the liver *GAMT* mRNA expression, and both the liver and muscle *CreaT* mRNA expression of birds. In this study, dietary GAA upregulated the mRNA expression of *GAMT* both in liver and muscle, but CrN supplementation linearly downregulated mRNA expression of *GAMT* both in liver and PM muscle. These results suggested that exogenous addition of GAA could promote the synthesis of creatine in the liver, but exogenous addition of CrN may inhibit self-synthesis of creatine via negative feedback on gene expression of *GAMT*. In spite of this, CrN supplementation linearly upregulated *CreaT* gene expression in PM muscle, promoted the absorption of creatine and the accumulation of PCr, which helps to improve cellular energy status of broiler muscle.

AMPK functions as a cell fuel gauge by sensing increased intracellular AMP/ATP ratio (Hardie, 2007). Therefore, AMPK is considered a central sensor of intracellular energy status to maintain cellular energy homeostasis. As a heterotrimeric protein, AMPK comprises of a catalytic α-subunit and 2 regulatory β- and γ- subunits (Hardie and Sakamoto, 2006). The α subunit has 2 isoforms, $\alpha 1$ and $\alpha 2$, which have different tissue expression. In mammals and chickens, *AMPKα1* is ubiquitously expressed, whereas *AMPKα2* is predominantly expressed in skeletal and cardiac muscle (Stapleton et al., 1996; Proszkowiec-Weglarz et al., 2006). AMPK is activated by upstream kinase *LKB1*, which phosphorylates AMPK at Thr172α Subunits (Woods et al., 2003). Ste20-related adaptor protein-α (**STRAD-α**) and mouse protein 25-α (**MO25-α**) are 2 accessory proteins of LKB1, MO25-α binds to the carboxyl terminal of *STRAD-α*, thereby stabilizing the association between *STRAD* and *LKB1* (Boudeau et al., 2003). Previous reports pointed out that ATP content decreased and the AMP/ATP ratio increased in contracting muscle lead to activation of AMPK (Hardie and Carling, 1997). Some previous studies have shown that AMPK also plays a significant role in the regulation of postmortem muscle glycolysis (Du et al., 2005; Xing et al., 2016). In our current study, lower relative mRNA expression of *LKB1* and *AMPKα2* in PM muscle of GAA_{600} and CrN groups accompanied by lower AMP/ATP ratio and higher concentration of creatine, PCr, and ATP suggested dietary addition of exogenous GAA and CrN could promote muscle energy status and inhibit the activation of LKB1/AMPK pathway.

CONCLUSIONS

To our knowledge this study is the first to evaluate CrN effects in broilers. We found that graded

supplementation with CrN at dose rate of 300 to 900 mg/kg to the broiler diets linearly promoted muscle energy status via strengthening the energy-buffering capacity of muscle creatine/PCr pool, linearly reduced muscle AMP/ATP ratio, and inhibited the activation of LKB1/AMPK pathway, which was conducive to improve meat quality by delaying postmortem glycolysis in muscle. Moreover, CrN showed better efficacy than GAA at the some dose. These results indicate that CrN may be a potential replacement for GAA as a new functional creatine supplement in poultry industry.

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DISCLOSURES

The authors declare that there are no conflicts of interest.

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