

Dietary guanidineacetic acid supplementation ameliorated meat quality and regulated muscle metabolism of broilers subjected to pre-slaughter transport stress by metabolomics analysis

Bolin Zhang,¹ Ning Liu, Kui Kang, Renbo Zhang, Meilin Hao, Peiyong Song, Qingrong Wang, Yuxiao Xie, and Chuntao Li

Department of Biology and Agriculture, Characteristic Laboratory of Animal Resources Conservation and Utilization of Chishui River Basin, Zunyi Normal College, Hong Huangang District, Zunyi 563006, People's Republic of China

ABSTRACT Pre-slaughter transport stress could induce multiple comprehensive variations in physiological and metabolic parameters of broilers. However, the entire metabolomics of pre-slaughter transport stress and supplementation of exogenous energy regulatory substances on broilers is still poorly understood. The metabolome characteristics of broilers subjected to 3 h pre-slaughter transport stress combined with 1,200 mg/kg guanidinoacetic acid (**GAA_{1,200}**) supplementation were analyzed using gas chromatography-mass spectrometry (**GC-MS**) in this study. The results showed that, compared to the control group (no transport), 3 h pre-slaughter transport stress (**T_{3h}**) decreased creatine (**Cr**), phosphocreatine (**PCr**) and adenosine triphosphate (**ATP**), and increased adenosine diphosphate (**ADP**), adenosine monophosphate (**AMP**) and the ratio of AMP to ATP in pectoralis muscle (**PM**) of broilers by high performance liquid chromatography (**HPLC**) analysis. However, **GAA_{1,200}** supplementation reversed the negative effects induced by 3 h pre-

slaughter transport stress. Besides, **GAA_{1,200}** supplementation elevated mRNA expression of creatine transporter in **PM**. Our metabolomics approaches demonstrated that 38 and 48 significant metabolites were separately identified between the control group and **T_{3h}** group, and **T_{3h}** group and 3 h pre-slaughter transport stress combined with **GAA_{1,200}** supplementation group using the standard of variable importance in the projection values >1 and $P < 0.05$. Among these, the metabolites involved in amino acid metabolism (alanine, glycine, serine, threonine, cysteine, methionine, phenylalanine, tyrosine, and tryptophan), oxidative stress (3-methylhistidine, 1-methylhistidine and glutathione), non-protein amino acid (citrulline) metabolism, and energy metabolism (**Cr**, **PCr**, sarcosine, and glycoamine) were confirmed through pathway enrichment analysis, which could be chosen as suitable candidate targets for further analysis of the effects of exogenous energy substances on broilers subjected to transport stress.

Key words: guanidine acetic acid, gas chromatography–mass spectrometry, muscle metabolomics, pre-slaughter transport stress, Qiandongnan Xiaoxiang chicken

2022 Poultry Science 101:101739

<https://doi.org/10.1016/j.psj.2022.101739>

INTRODUCTION

Qiandongnan Xiaoxiang chicken, a local excellent broiler breeder, which is largely raised in southeast of Guizhou province and is characteristics by small-sized, yellow-brown flat feathers, and black-colored bones. Qiandongnan Xiaoxiang chicken, a slow-growth native breeder, is usually raised for 5 to 6 months before it is sold on the market. Moreover, the

meat of Qiandongnan Xiaoxiang chicken is tender and is characterized by the nutrition value of much protein and lower fat (Liu et al., 2020). However, intensively reared broiler chickens are inevitably transported from farms to slaughter plants when they reach the desired slaughter body weight (Zhang et al., 2019b). The journey of broilers from farm to the processing plant exposed to multidimensional stressors, such as catching, crating, feed and water withdrawal, and transport, may lead to negative effects on animal welfare and the alterations in various physiological and metabolic parameters of broilers (Zhang et al., 2017b; Hussnain et al., 2020), which included the elevated plasma corticosterone concentrations (Zhang et al., 2009), the increased heterophil to lymphocyte ratio (Yalcin and Guler, 2012), and

© 2022 The Authors. Published by Elsevier Inc. on behalf of Poultry Science Association Inc. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

Received September 1, 2021.

Accepted January 13, 2022.

¹Corresponding author: bolin-zhang@163.com

the altered meat fatty acid profile (Zanetti et al., 2011). Under the conditions of pre-slaughter transport, energy consumption was accelerated and a rapid glycolysis of meat was initiated, as consequences of lactic acid accumulation in muscle and the production of an inferior chicken meat (Zhang et al., 2017a). Moreover, it had been demonstrated that pre-slaughter stress was considered as one of the important factors contributing to an increase in incidences of pale, soft, exudative (PSE)-like chicken meat, characterized by lighter appearance, softer texture, lower water holding capacity, excessive yield losses, and formation of soft gels, which can cause major economic losses to poultry enterprises (Wang et al., 2017; Zhang et al., 2021). Therefore, it is important to take nutritional measures to ameliorate body energy storage in order to attenuate stress-induced muscle energy expenditure, which may contribute to improving meat quality.

Guanidinoacetic acid (GAA), formed from arginine and glycine catalyzed by L-arginine: glycine amidinotransferase (AGAT), acts as a natural precursor of creatine (Cr) and its phosphorylated derivative, phosphocreatine (PCr). PCr, a high-energy molecule, was able to fuel cellular activities and moderate the accumulation of adenosine diphosphate (ADP) from adenosine triphosphate (ATP) during high rates of cellular metabolism (Wallimann et al., 2011; Tossenberger et al., 2016). The formation of GAA catalyzed by AGAT primarily took place in kidney and pancreas (Ostojic, 2015). GAA was then transported to the liver for Cr biosynthesis catalyzed by S-adenosyl-L-methionine: N-guanidino-acetate methyltransferase (GAMT; Zhang et al., 2019b). Subsequently, Cr was transported into muscle and to other target cells via the creatine transporter (CrT) against a concentration gradient and could be involved in energy metabolism through the Cr and PCr system (Liu et al., 2015). Numerous studies suggested that GAA supplementation could improve energy status of the muscle (Michiels et al., 2012; McBreairey et al., 2015), as evidenced by the increased available energy reserve such as PCr and ATP, which contributed to delaying the post-mortem muscle pH decline (Li et al., 2018). In our previous study, dietary 1,200 mg GAA supplementation elevated the concentrations of ATP and Cr in muscle, and ameliorated postmortem glycolysis and energy metabolism (Zhang et al., 2021). Although the variations in energy metabolism of broilers subjected to pre-slaughter transport stress were available, to the best of our knowledge, the entire metabolomics of GAA supplementation on broilers subjected to pre-slaughter transport stress is still poorly understood.

We thus speculate that dietary supplemented with GAA could improve postmortem meat quality by regulating muscle metabolism. Therefore, the aim of this study was to identify potential predictive biomarkers and to elucidate the metabolic variations in broilers by characterizing the whole metabolomics signature of

broilers in response to GAA supplementation comprising pre-slaughter transport stress.

MATERIALS AND METHODS

Diets and Experiment Design

All the procedures involving animal care and experiment treatments were approved by the Institutional Animal Care and Use Committee of Zunyi Normal College. The basal diet was formulated to meet the nutrient requirements of Nutrient Requirements of Chinese Meat-Type Yellow Feathered Chickens. The ingredient composition and nutrient levels of diets are shown in Table 1.

All broilers were fed with the same commercial diets from 1 d of age to 22-wk-old. One hundred and twenty Qiangongnan Xiaoxiang chickens of 22-wk-old (provided by Guizhou Rongjiang Shannong Development Co., Ltd, China) were chosen and randomly assigned into 3 groups (A, B, and C), with 20 replicates per group and 2 broilers per replicate. Broilers in group A and group B were fed with the basal diet, while group C received diets containing 1,200 mg/kg GAA based on the basal diet for 15 d. During the period of the experiment, all birds had free access to the feed in mash form and fresh water. GAA (>99% purity) was purchased from Tianjin Tiancheng Pharmaceutical Co. Ltd. (Tianjin, China) and the amount of GAA added to the diets was according to our previous study (Zhang et al., 2021).

Transportation

At d 15, after 8 h feed forbidden without water withdrawal before pre-slaughter transportation, all broilers were treated according to the following procedures: group B and group C were transported for 3 h at a speed of 80 km/h, however, group A were treated with no transportation (refined as the control). During the

Table 1. Composition and nutrient levels of the basal diet (air-dry basis, %).

Items	Contents	Nutrient levels	Contents
Corn	58.62	Crude protein ² (%)	19.05
Soybean meal	25.00	Metabolic energy (MJ/kg)	12.56
Corn gluten meal	3.05	Lysine (%)	0.98
Rapeseed meal	5.63	Methionine (%)	0.72
Soybean oil	3.93	Calcium (%)	0.90
L-Lysine HCl	0.32	Available phosphorus (%)	0.40
DL-Methionine	0.10		
Calcium monophosphate	1.61		
Limestone	1.18		
Salt	0.15		
1% Premix ¹	1.00		

¹Premix provided the following per kg of the diet: vitamin A 10,000 IU; vitamin D3 2,000 IU; vitamin E 25 mg; Vitamin K, 2.8 mg; thiamine 2.50 mg; riboflavin 7.5 mg; nicotinamide 40 mg; calcium pantothenate, 25 mg; pyridoxine-HCl, 3 mg; biotin, 0.20 mg; folic acid, 1.5 mg; vitamin B12, 0.015 mg; ferrous (as ferrous sulfate) 80 mg; copper (as copper sulfate) 8 mg; manganese (as manganese sulfate) 100 mg; zinc (as zinc sulfate) 60 mg; iodine (as potassium iodide) 0.35 mg; selenium (as sodium selenite) 0.3 mg

²The crude protein level was the measurement value and others were calculated values.

transportation, ten birds within the same replicate were placed into one crate (0.73 m × 0.54 m × 0.26 m), and 8 crates were randomly distributed in the same truck. The temperature and humidity during the 3 h transportation was separately 27.0 to 32.6°C and 77.8 to 87.8%.

Sample Collection

After 3 h transportation and rest for 1 h, 8 broilers with a body weight close to the mean body weight in each group were chosen. Appropriate 10 mL blood of each bird was collected into tubes coated with EDTA via the wing vein to separate the plasma for subsequent analysis. The birds were then slaughtered via exsanguination after electrical stunning (50 V: alternating current, 400 Hz for 5 s each one). Immediately after death, the carcasses were soaked in hot water ($60 \pm 0.5^\circ\text{C}$) for 30 s, and then introduced into a poultry plucker for 20 s (Zhang et al., 2019b). After being defeathered mechanically, the entire left pectoralis muscle (PM) was stored at 4°C for meat quality analysis. About 10 g of the right PM and liver were separately collected into tube, and stored in liquid nitrogen for further analysis.

Growth Performance

Broilers for each replicate were separately weighted to calculate the average daily weight gain (ADG) at the beginning and the end of the trial. And feed consumption for each replicate was also recorded daily to calculate the average daily feed intake (ADFI) and the ratio of feed to gain (F/G).

Plasma Analysis

The concentration of glucose in plasma was analyzed using a commercial test kit (Nanjing Jiancheng Bioengineering Co., Ltd., Nanjing, China). The concentration of corticosterone (CORT) was measured with a commercial test kit in accordance with manufacturer's instructions (Cusbio Biotech. Co., Ltd., Wuhan, China).

Meat Quality Measurements

The pH values of postmortem muscles at 45 min ($\text{pH}_{45\text{min}}$) and 24 h ($\text{pH}_{24\text{h}}$) were measured at 3 locations using a digital portable pH meter (PHBF-260; Shanghai Instrument Electric Science Instrument Co., Ltd., Shanghai, China), respectively. The values of meat color of the right PM at postmortem 24 h, containing L^* (lightness), a^* (redness), and b^* (yellowness), were determined using a portable spectrophotometer (YS3010; Shenzhen San'enshi Technology Co., Ltd., Shenzhen, China). After storage at 4°C for 24 h, the measurement of drip loss, cooking loss, and shear force of PM muscles were conducted according to our previous study (Zhang et al., 2021).

Determination of Cr, PCr, and Adenosine Nucleotides in PM

The concentrations of Cr and PCr, combined with the contents of ATP, ADP, and AMP in PM, were determined by high performance liquid chromatography (HPLC) according to a previous reported method with some modification (Liu et al., 2015; Zhang et al., 2021). Frozen PM samples were taken from liquid nitrogen and immediately collected into tubes which contained 2 mL 5% ice-cold perchloric acid. After homogenized for 1 min and standing in an ice bath for 15 min, the supernatants of homogenates were collected after centrifugation at 15,000 g at 4°C for 10 min. The pH of the supernatants was adjusted to 6.5 with 1.03 M KOH for adenosine nucleotides analysis and to 7.0 with 0.8 M K_2CO_3 for Cr and PCr analysis, respectively. The obtained supernatants were filtered through a 0.45 μm membrane, then followed by an injection into Alliance 2695 HPLC system (Water Corporation, Milford, MA), which was equipped with a Waters SunFire C18 column (250 mm × 4.6 mm, 5 μm) at a temperature of 25°C for Cr and PCr determination, and 30°C for ATP, ADP, and AMP analysis. The ultraviolet wavelengths for Cr and PCr determination and the analysis of ATP, ADP, and AMP were 210 nm and 245 nm, respectively. A mixture of methyl cyanides and 29.4 mM KH_2PO_4 buffer (2:98, volume ratio) and a mixture of methanol and phosphate buffer (13.5:86.5, volume ratio) were separately used as the mobile phase for the determination of Cr and PCr and the analysis of ATP, ADP, and AMP.

Metabolomic Sample Preparation

20 mg samples were accurately weighed and transferred to a 1.5-mL Eppendorf tube, containing 20 μL of 2-chloro-l-phenylalanine as internal standard dissolved in methanol (0.3 mg/mL) and 600 μL of methanol/water extraction solvent (4:1, v/v). Subsequently, samples mentioned above were stored at -80°C for 2 min, and were grinded at 60 HZ for 2 min. Afterward, 120 μL of chloroform was added to each sample, followed by 10 min ultrasound-associated extraction at ambient temperature and 10 min storage at 4°C. A quality control (QC) sample was prepared by mixing aliquots of all samples as a single pooled sample. About 250 μL supernatants were transferred to a glass sampling vial for vacuum-dry at room temperature, mixed with 80 μL methoxylamine hydrochloride (dissolved in pyridine, 15 mg/mL) for 2 min and incubated at 37°C for 90 min. Subsequently, 80 μL N,O-bis(trimethylsilyl) trifluoroacetamide BSTFA and 20 μL n-hexane were added into the mixture. The prepared mixture mentioned above was derivatized at 70°C for 60 min. The samples were placed at ambient temperature for 30 min before analyzing using GC-MS.

GC-MS Analysis

GC-MS analysis was performed based on a previous study with moderate modification (Lu et al., 2018), using

an Agilent 7890B gas chromatography system coupled to an Agilent 5977A MSD system (Agilent Technologies Inc., CA). Samples were injected onto a DB-5MS fused-silica capillary column (30 m × 0.25 mm × 0.25 μm, Agilent J & W Scientific, Folsom, CA) to separate the derivatives. Helium with purity >99.999% was used as the carrier gas at a constant flow rate of 1 mL / min and the injector temperature was maintained at 260°C. The injection volume was 1 μL by splitless mode. The initial oven temperature was 60°C, ramped to 125°C at a rate of 8°C/min, to 210°C at a rate of 5°C/min into 270°C at a rate of 10°C/min, to 305°C at a rate of 20°C/min, and finally held at 305°C for 5 min. The temperature of MS quadrupole and ion source (electron impact) was set to 150°C and 230°C, respectively. The collision energy was 70 eV. Mass spectrometric data was acquired in a full-scan mode (m/z 50–500). The QCs were injected at regular intervals (every 8 samples) throughout the analytical run to provide a set of data from which repeatability could be assessed.

Real-Time Quantitative RT-PCR Analysis

RNA extraction of PM samples frozen in liquid nitrogen was conducted using Trizol (Invitrogen, Carlsbad, CA). Subsequently, the extracted RNA contents were determined using a spectrophotometer (Nanodrop ND-1000, Thermo Fisher Scientific, Wilmington, DE). After DNA removal using DNase I (TaKaRa, Biotechnology CO. Ltd., Dalian, China), the purified RNA was reverse transcribed into cDNA and real-time PCR was conducted with PrimeScript RT master mix and SYBR premix Ex Taq kits (TaKaRa, Biotechnology CO. Ltd.), respectively. The real-time PCR program consisted of one cycle at 95°C for 30 s, 40 cycles at 95°C for 5 s and 60°C for 30 s. All samples were run in triplicate and the expression of target genes relative to the house keeping gene (β -actin) were calculated using $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The primer sequences for RT-PCR analysis shown in Table 2.

Statistical Analysis

Analysis base file converter software (ChemStation, version E.02.02.1431, Agilent) was used to convert the raw data and ChromaTOF (version 4.34; LECO, St Joseph, MI) was used to analyze the data. Principle component analysis (PCA) and an orthogonal partial least-squares-discriminant analysis (OPLS-DA) were performed to visualize the metabolic differences among experimental groups. The Hotelling's T² region, shown as an ellipse in score plots of the models, defines the 95% confidence interval of the modeled variation. Variable importance in the projection (VIP) ranks the overall contribution of each variable to the OPLS-DA model, and those variables with VIP > 1 are considered relevant for group discrimination. The differential metabolites were selected on the basis of the combination of a statistically significant threshold of variable influence on projection VIP values obtained from the OPLS-DA model and P

values from a two-tailed Student's t test on the normalized peak areas from different groups, where metabolites with VIP >1.0 and $P < 0.05$ were considered as differential metabolites, which were identified and performed by functional enrichment analysis using MetaboAnalyst 3.0 website according to the previous reports (Xia et al., 2015; Boerboom et al., 2018; Wu et al., 2021).

Data analysis for concentrations of Cr, PCr, adenosine nucleotides in muscle, combined with mRNA expression in muscle and liver, were conducted using one-way analysis of variance (ANOVA) with the SPSS statistical software (version 20.0, SPSS Institute, Inc., Chicago, IL). The results are presented with mean values with standard error of the mean (SEM), and $P < 0.05$ was considered statistically significant.

RESULTS

Growth Performance

As shown in Table 3, compared with broilers in Group A and Group B (fed with the basal diet), dietary supplemented with 1,200 mg/kg GAA (Group C) did not affect ADG, ADFI, or F/G of broilers ($P > 0.05$).

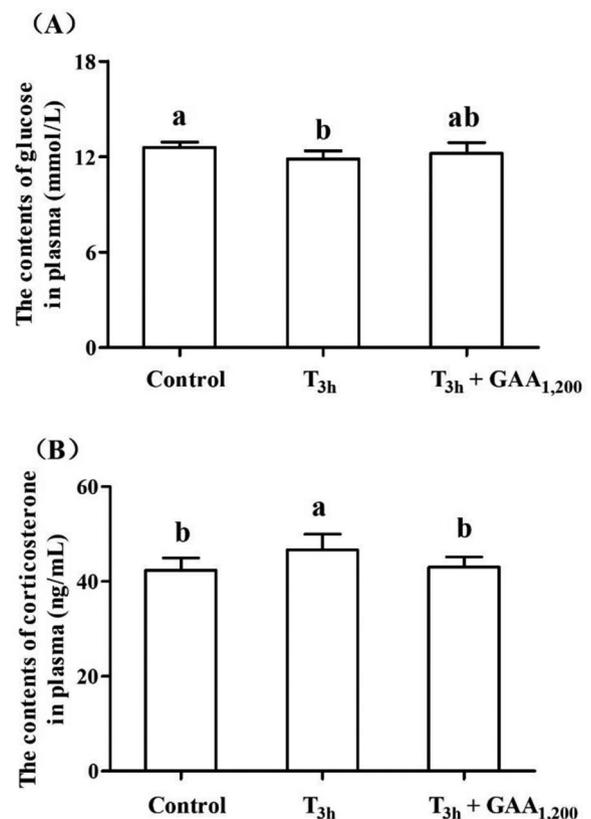


Figure 1. Effects of dietary guanidinoacetic acid (GAA) supplementation on the concentrations of glucose (A) and corticosterone (B) in plasma of Qiantongnan Xiaoxiang chickens subjected to pre-slaughter transport stress ($n = 8$ per treatment). Results are expressed as the mean value and standard deviation. Means within different letters differ significantly ($P < 0.05$). Control, broilers fed the basal diet with no transportation; T_{3h}, broilers fed the basal diet and experienced a 3 h pre-slaughter transportation; T_{3h}+GAA_{1,200}, broilers fed the basal diet supplemented with GAA at 1,200 mg/kg and experienced a 3 h pre-slaughter transportation.

Table 2. Primer Sequences used for real-time PCR.

Genes	Primers (5'→3')	Product size	Gene accession no.
GAMT	Sense: ACCAGTTTGCCTTCATCA Antisense: CTCTATGTCGCTGTATTTCG	119 bp	XM 001234062.3
¹ CrT	Sense: TGAACACTACAAACCGCTGACG Antisense: GCTCGTAGATAACGGTGCAG	120 bp	JN628439.2
² β -actin	Sense: ATGTCCACCGCAAATGCTTC Antisense: AAATAAAGCCATGCCAATCTCGTC	113 bp	NM_205518.1

Abbreviations: CrT, creatine transporter; GAMT, S-adenosyl-L-methionine: guanidinoacetate N-methyltransferase.

¹Means not followed by the same letter differ ($P < 0.050$).

²The primer sequence of β -actin was designed according to our previous study (Zhang et al.,2021).

Table 3. Effects of guanidine acetic acid supplementation on growth performance of Qiandongnan Xiaoxiang chickens.

Items	Group A	Group B	Group C	SEM	P value
ADG (g/d)	29.74	30.08	30.43	0.524	0.886
ADFI (g/d)	94.89	95.73	93.33	1.248	0.765
F/G (g/g)	3.19	3.20	3.07	0.068	0.718

Results are expressed as the mean value (n = 20 per treatment) and standard error of the mean (SEM).

Broilers in Group A and Group B were fed with the basal diet, while broilers in Group C were fed with diet supplemented with 1,200 mg/kg guanidine acetic acid based on the basal diet.

Abbreviations: ADG, average daily weight gain; ADFI, average daily feed intake; F/G, the ratio of feed to gain.

Plasma Parameter

As illustrated in Figure 1, 3 h pre-slaughter transport stress increased the concentration of corticosterone (Figure 1B) in plasma in comparison with the control group (no transport stress, $P < 0.05$). In contrast, broilers subjected to 3 h pre-slaughter transport stress showed lower glucose concentration in plasma than those in the control group (Figure 1A, $P < 0.05$). However, compared with the control group, dietary supplemented with 1,200 mg/kg GAA decreased the content of corticosterone in plasma ($P < 0.05$).

Meat Quality Analysis

According to Table 4, broilers in 3 h pre-slaughter transport stress showed lower pH_{24h} and higher L* value, drip loss and cooking loss of the PM muscle than those in the control group ($P < 0.05$). But in comparison with

T_{3h} group, dietary supplemented with 1,200 mg/kg GAA increased pH_{24h} and decreased L* value, drip loss and cooking loss of the PM muscle ($P < 0.05$). There were no differences in pH_{45min}, a*, b* and shear force in the PM muscle among these three treatments ($P > 0.05$).

Concentrations of Cr, PCr, ATP, ADP, and AMP in PM

As shown in Table 5, compared with the control group, 3 h pre-slaughter transport decreased the contents of Cr and ATP in PM ($P < 0.05$). In contrast, 3 h pre-slaughter transport increased the contents of ADP, AMP, and the ratio of AMP: ATP ($P < 0.05$). However, there were no differences in the ratio of PCr:Cr and the concentration of PCr between 3 h pre-slaughter transport and the control group ($P > 0.05$). Notably, compared with 3 h pre-transport stress, 1,200 mg GAA supplementation resulted in the increases in the contents of PCr and ATP in PM ($P < 0.05$).

Table 4. Effects of GAA supplementation on meat quality of the pectoralis major muscle of Qiandongnan Xiaoxiang chickens experienced pre-slaughter transport stress.

Items	Control group	T _{3h}	T _{3h} + GAA _{1,200}	SEM	P value
pH _{45min}	6.37	6.36	6.39	0.014	0.790
pH _{24h}	5.82 ^a	5.69 ^b	5.85 ^a	0.017	<0.001
L*	51.97 ^b	55.96 ^a	52.22 ^b	0.601	0.005
a*	1.24	1.40	1.28	0.043	0.298
b*	5.41	5.93	5.08	0.211	0.259
Drip loss (%)	2.26 ^b	2.73 ^a	2.23 ^b	0.072	0.003
Cooking loss (%)	17.38 ^b	18.27 ^a	17.02 ^b	0.198	0.020
Shear force (N)	24.36	24.79	24.47	0.099	0.175

Results are expressed as the mean value and standard error of the mean (SEM).

^{ab}Means within the same row with different superscripts differ significantly (n = 8 per treatment, $P < 0.05$). Control group, broilers fed the basal diet with no transportation; T_{3h}, broilers fed the basal diet and experienced a 3 h transportation; T_{3h}+GAA_{1,200}, broilers fed the basal diet supplemented with GAA at 1,200 mg/kg and experienced a 3 h transportation. Abbreviations: GAA, guanidine acetic acid; pH_{45min}, pH at 45 min postmortem; pH_{24h}, pH at 24 h postmortem; L*, lightness; a*, redness; b*, yellowness.

Table 5. Effects of GAA supplementation on muscle energy status in muscle of Qiandongnan Xiaoxiang chickens experienced pre-slaughter transport stress.

Items	Control group	T _{3h}	T _{3h} +GAA _{1,200}	SEM	P value
Cr	22.40 ^b	20.18 ^c	24.73 ^a	0.602	0.001
PCr	2.36 ^{ab}	2.19 ^b	2.47 ^a	0.045	0.022
PCr : Cr	0.11	0.11	0.10	0.003	0.413
ATP	3.52 ^a	3.03 ^b	3.38 ^a	0.062	<0.001
ADP	0.95 ^b	1.11 ^a	0.97 ^b	0.021	<0.001
AMP	0.34 ^b	0.46 ^a	0.36 ^b	0.016	0.012
AMP : ATP	0.10 ^b	0.15 ^a	0.11 ^b	0.007	<0.001

Results are expressed as the mean value and standard error of the mean (SEM).

^{ab}Means within the same row with different superscripts differ significantly (n = 8 per treatment, $P < 0.05$). Control group, broilers fed the basal diet with no transportation; T_{3h}, broilers fed the basal diet and experienced a 3 h transportation; T_{3h}+GAA_{1,200}, broilers fed the basal diet supplemented with GAA at 1,200 mg/kg and experienced a 3 h transportation. Abbreviations: ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate; Cr, creatine; GAA, guanidine acetic acid; PCr, phosphocreatine.

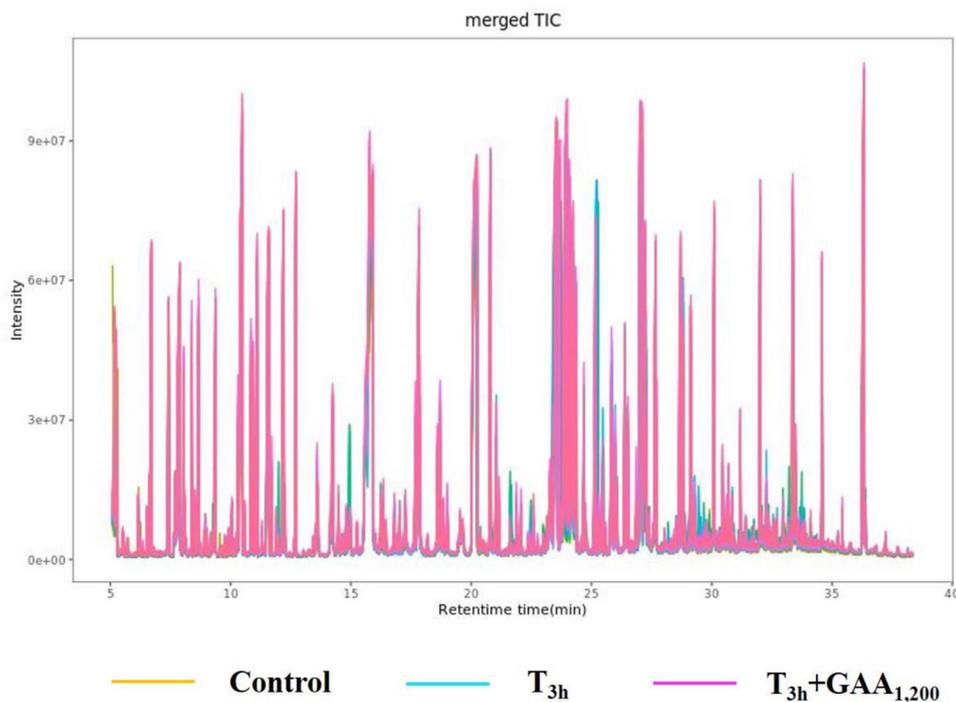


Figure 2. Total ion chromatograms (TIC) of pectoralis muscle from broilers. Control, broilers fed the basal diet with no transportation; T_{3h} , broilers fed the basal diet and experienced a 3 h pre-slaughter transportation; $T_{3h}+GAA_{1,200}$, broilers fed the basal diet supplemented with GAA at 1,200 mg/kg and experienced a 3 h pre-slaughter transportation.

Furthermore, 1,200 mg GAA supplementation lowered the contents of ADP, AMP and the ratio of AMP:ATP in comparison with broilers subjected to 3 h pre-slaughter transport ($P < 0.05$). However, there were no differences in the ratio of PCr to Cr among these three treatments ($P > 0.05$).

Metabolic Profiles in PM

Representative total ion chromatograms (TIC) of PM samples from broilers are presented in Figure 2. After pretreatment and standardization, 407 effective

peaks and 240 metabolites were identified in the test samples following extraction ion chromatography.

PCA Analysis

The score plots of PM of broilers in the control group, T_{3h} group and $T_{3h}+GAA_{1,200}$ group by GC-MS are shown in Figure 3. There was an obvious separation between the T_{3h} group and the other 2 groups, indicating that 3 h pre-slaughter transportation induced significant metabolic variations in postmortem PM. This method also enabled detection and exclusion of any outliers, defined as observations located outside the 95% confidence region of the model.

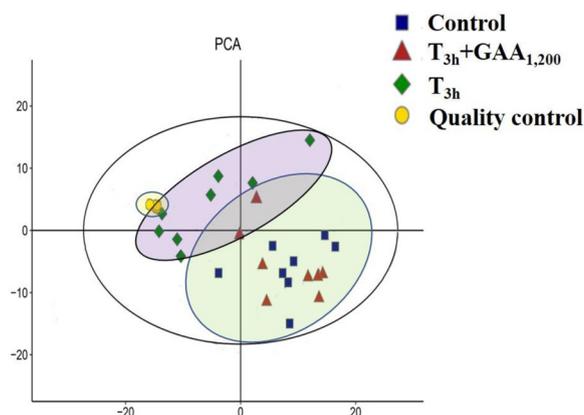


Figure 3. Principal component analysis (PCA) score plots derived from GC-MS profiles of pectoral muscle of broilers in the control group (no transportation), 3 h pre-slaughter transportation (T_{3h}) group and 3 h pre-slaughter transportation combined with 1,200 mg/kg guanidine acetic acid supplementation ($T_{3h}+GAA_{1,200}$) group.

Orthogonal Projections Latent Structures Discriminant Analysis

OPLS-DA was performed to better understand the different metabolic profiles of PM obtained from broilers in the control group vs. the T_{3h} group (Figure 4A) and the T_{3h} group and the $T_{3h}+GAA_{1,200}$ group (Figure 4B). Besides, there was an obvious separation between the control group and the T_{3h} group, as well as between the T_{3h} group and the $T_{3h}+GAA_{1,200}$ group, indicating that significant changes occurred in the metabolic profiles of PM. The model parameters for the control group vs. the T_{3h} group were $R^2Y(\text{cum}) = 0.987$ and $Q^2(\text{cum}) = 0.674$, suggesting that the predictive ability of the model was 0.674 and that 98.7% of the samples conformed to the established model. Similarly, the model parameters for the T_{3h} group vs. the

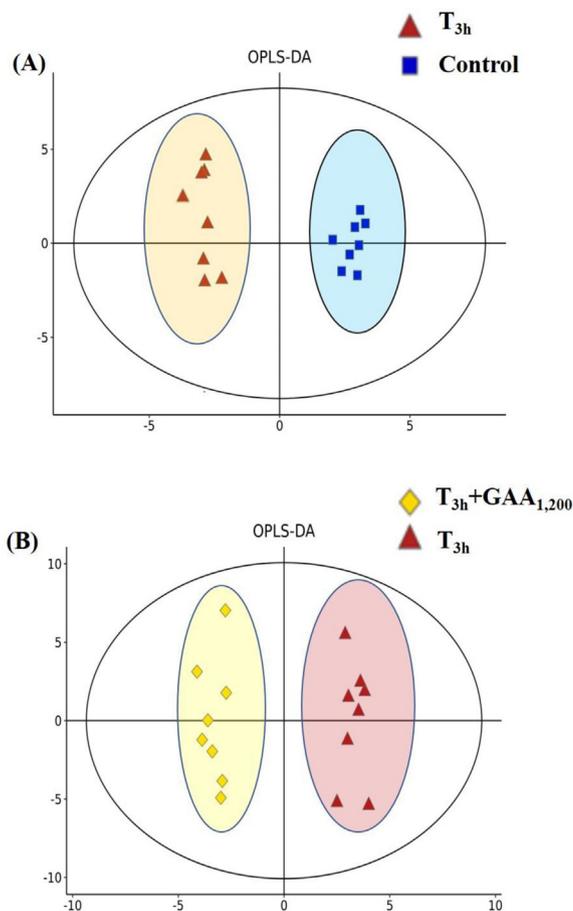


Figure 4. Orthogonal projections to latent structure-discriminant analysis (OPLS-DA) score plots following GC-MS profiles of pectoral muscle (PM) of broilers obtained from the control group (no transportation) and 3 h pre-slaughter transportation group (A), and 3 h pre-slaughter transportation and the 3 h pre-slaughter transportation combined with 1,200 mg/kg guanidine acetic acid supplementation (B). The descriptive and predictive performance characteristics of the models are $R^2Y(\text{cum}) = 0.987$, $Q^2(\text{cum}) = 0.674$ for the control group and T_{3h} group, $R^2Y(\text{cum}) = 0.979$, $Q^2(\text{cum}) = 0.747$ for T_{3h} and $T_{3h}+GAA_{1,200}$ group.

$T_{3h}+GAA_{1,200}$ group were $R^2Y(\text{cum}) = 0.979$ and $Q^2(\text{cum}) = 0.747$, demonstrating that the predictive ability of the model was 0.747 and that 97.9% of the samples conformed to the established model.

Screening of Discriminate Metabolites

Multidimensional analysis and single dimensional analysis were used to screen the different metabolites identified by the OPLS-DA model. The screening conformed to the criteria of the first principal component of the OPLS-DA model with variable important in $VIP > 1$ and t test with $P < 0.05$. Moreover, fold change analysis was done to evaluate the specific changes in the discriminated metabolites in PM samples obtained from the T_{3h} group compared with those from the control group and the $T_{3h}+GAA_{1,200}$ group. Thirty-eight metabolites with significant different levels were identified between the control group and the T_{3h} group (Table 6). Meanwhile, 48 metabolites with significant different levels were found between the T_{3h} group and the $T_{3h}+GAA_{1,200}$ group

Table 6. Significant metabolites in muscle of broilers between the control group and T_{3h} group.

Metabolites	Retaining time (min)	VIP	P value	Change
Pyruvic acid	6.51	1.06	<0.05	↑
L-Lactic acid	6.69	1.31	<0.01	↑
Cytosin	7.08	1.39	<0.01	↓
L-Alanine	7.42	1.33	<0.01	↓
Creatine	7.85	1.75	<0.05	↓
3-hydroxybutyric acid	8.38	1.46	<0.01	↑
Glutathione	8.57	1.05	<0.05	↓
N-methylalanine	8.58	1.84	<0.01	↑
β -alanine	8.94	1.30	<0.01	↓
Succinic acid	9.41	1.48	<0.01	↑
1-methylhydantoin	9.55	3.21	<0.01	↓
Homoserine	10.11	1.20	<0.01	↑
Dopamine	10.61	1.24	<0.05	↑
Glucosamine	10.66	1.31	<0.01	↑
Trans-4-hydroxy-L-proline	10.78	2.12	<0.05	↓
Glycine	11.09	1.37	<0.05	↓
Hydrocinnamic acid	13.26	1.53	<0.05	↓
Arbutin	14.17	1.79	<0.05	↓
Creatine phosphate	14.55	1.02	<0.05	↓
Noradrenaline	14.92	2.16	<0.01	↑
Malate	14.93	2.49	<0.05	↑
Phosphoglycolic acid	17.23	2.19	<0.01	↑
Allantoic acid	17.24	2.00	<0.01	↑
O-phosphoethanolamine	21.15	2.48	<0.01	↑
3-methylhistidine	21.59	2.20	<0.05	↑
Hypoxanthine	21.61	2.05	<0.05	↑
Myristic acid	22.59	1.06	<0.05	↑
Glucose	23.71	1.02	<0.05	↓
Pantothenic acid	25.31	1.09	<0.05	↓
Palmitic acid	26.38	1.27	<0.01	↑
Trehalose-6-phosphate	26.62	2.30	<0.05	↑
N-acetyl-D-glucosamine	26.86	1.27	<0.05	↓
Allose	27.70	1.82	<0.01	↓
Linoleic acid	28.72	1.18	<0.05	↑
D-myo-inositol 4-phosphate	30.85	1.31	<0.05	↑
Cis-gondoic acid	30.94	1.16	<0.05	↑
Zymosterol	36.60	1.28	<0.05	↑
Lanosterol	38.21	1.10	<0.05	↑

VIP, Variable important in projection; change, the ratio of mean peak area of significant differential metabolites of broilers in 3 h pre-slaughter transport to the ratio of mean peak area of significant differential metabolites of broilers in the control group; ↑, the ratio of the concentration of significant metabolites in 3 h pre-slaughter transportation group to those in the control group was more than 1; ↓, the ratio of the concentration of significant metabolites in the 3 h pre-slaughter transportation group to those in the control group was less than 1.

(Table 7). Among the discriminated metabolites between the control and the T_{3h} group, the concentrations of 23 metabolites were higher in the T_{3h} group than in the control group, and 15 metabolites were lower than in the control group. As shown in Table 7, among the metabolites with significantly different levels between the T_{3h} group and the $T_{3h}+GAA_{1,200}$ group, the concentrations of 36 metabolites were higher and 12 metabolites were lower in broilers in the $T_{3h}+GAA_{1,200}$ group than in the T_{3h} group.

Pathway Enrichment Analysis

Pathway enrichment analysis of all the discriminating metabolites identified by the 2 OPLS-DA models was carried out based on the KEGG database. There were 16 significant metabolic pathways between the control group and the T_{3h} group (Figure 5A), combined with

Table 7. Significant metabolites in muscle of broilers between T_{3h} group and T_{3h} + GAA₁₂₀₀ group.

Metabolites	Retaining time (min)	VIP	P value	Change
Pyruvic acid	6.51	1.55	<0.01	↓
L-Lactic acid	6.69	1.20	<0.05	↓
Sarcosine	7.04	1.38	<0.05	↑
Creatine	7.85	1.26	<0.05	↑
3-Hydroxybutyric acid	8.38	1.07	<0.01	↑
Succinic acid	9.41	1.21	<0.01	↓
1-Methylhydantoin	9.55	2.38	<0.01	↓
Ethanolamine	10.30	1.62	<0.01	↑
Glycine	11.09	1.63	<0.01	↑
Uracil	11.71	1.14	<0.05	↑
D-Erythro-sphingosine	12.23	1.82	<0.01	↓
L-Threonine	12.71	1.08	<0.01	↑
Putrescine	13.21	1.30	<0.01	↑
5-Aminovaleric acid	13.43	1.01	<0.01	↑
Arbutin	14.17	1.66	<0.01	↑
Creatine phosphate	14.55	1.02	<0.01	↑
Nicotinamide	14.80	1.19	<0.01	↑
Noradrenaline	14.92	1.70	<0.01	↑
L-Methionine	15.65	1.44	<0.01	↑
Tartaric acid	15.68	1.23	<0.01	↑
L-2-Hydroxyglutaric acid	16.81	1.67	<0.05	↓
Glycocyanine	16.91	1.97	<0.01	↑
Phosphoglycolic acid	17.23	1.85	<0.01	↑
Allantoic acid	17.24	1.03	<0.01	↑
Allantoin	17.26	1.20	<0.05	↑
L-Phenylalanine	17.86	1.17	<0.05	↓
3,4-Dihydroxybenzoic acid	18.47	2.39	<0.01	↑
D-Xylitol	19.64	1.07	<0.05	↑
Amino adipic acid	19.96	1.62	<0.01	↑
Pseudo uridine	21.30	1.15	<0.05	↑
Hypoxanthine	21.61	1.90	<0.01	↓
Citrulline	22.07	1.61	<0.01	↓
Cadaverine	22.77	1.77	<0.05	↑
Adenine nucleotides	23.95	1.88	<0.05	↓
L-Histidine	23.95	1.81	<0.05	↑
L-Lysine	24.07	1.23	<0.05	↑
2'-Deoxyguanosine	24.28	1.68	<0.05	↑
Saccharopine	24.56	2.30	<0.01	↑
Pantothenic acid	25.31	1.48	<0.01	↑
D-Ribose 5-phosphate	27.31	2.09	<0.01	↑
Allose	27.70	1.71	<0.01	↑
L-Cystathionine	27.84	1.88	<0.05	↑
L-Tryptophan	28.68	1.07	<0.05	↑
Raffinose	29.58	1.44	<0.01	↑
L-Cystine	29.64	1.70	<0.05	↑
Pyridoxine	31.01	1.10	<0.05	↓
Zymosterol	36.60	1.18	<0.05	↓
5'-Deoxy-5'-methylthioadenosine	38.26	2.26	<0.01	↑

VIP, Variable important in projection; change, the ratio of mean peak area of significant differential metabolites of broilers in T_{3h}+GAA_{1,200} group to the ratio of mean peak area of significant differential metabolites of broilers in T_{3h} group; ↑, the ratio of the concentration of significant metabolites in T_{3h}+GAA_{1,200} group to those in T_{3h} group was more than 1, ↓, the ratio of the concentration of significant metabolites in T_{3h}+GAA_{1,200} group to those in T_{3h} group was less than 1.

T_{3h}, broilers fed the basal diet and experienced a 3 h transportation; T_{3h}+GAA_{1,200}, broilers fed the basal diet supplemented with GAA at 1,200 mg/kg and experienced a 3 h transportation.

the T_{3h} group and the T_{3h}+GAA_{1,200} group (Figure 5B), respectively. The best-represented pathways between the control group and the T_{3h} group recognized by the discriminating metabolites were ABC transporters, neuroactive ligand-receptor interactions, glyoxylate and dicarboxylate metabolism, gap junctions, carbon metabolism, purine metabolism, citrate cycle (TCA cycle), cysteine and methionine metabolism, alanine, aspartate and glutamate metabolism, pantothenate and CoA biosynthesis, pyruvate metabolism, biosynthesis of

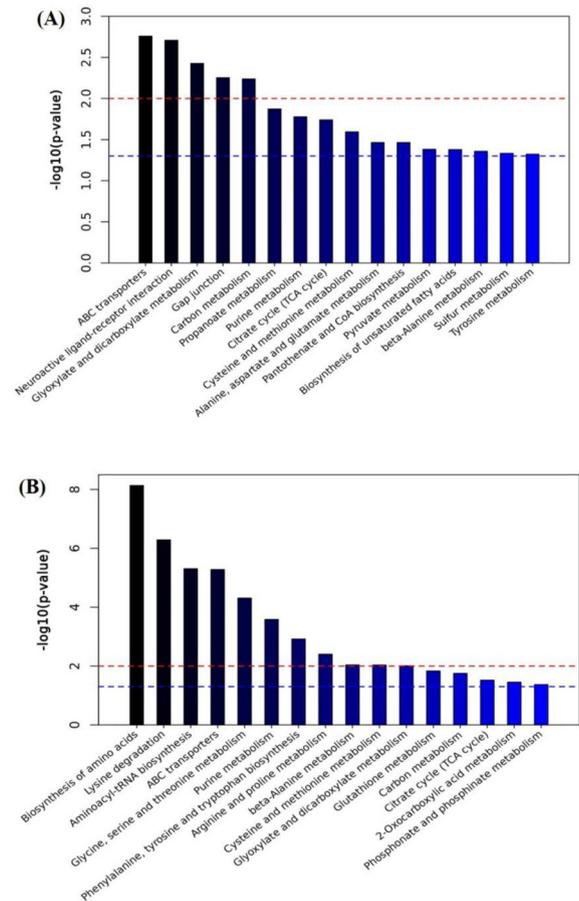


Figure 5. Metabolite pathway enrichment analysis was performed using the discriminating metabolites identified by the two individual orthogonal projections to latent structures discriminant analyses models. Sixteen representative metabolic pathways were obtained from the control group (no transportation) and 3 h pre-slaughter transportation group (A), and 3 h pre-slaughter transportation and the 3 h pre-slaughter transportation combined with 1,200 mg/kg guanidine acetic acid supplementation (B), respectively. The ordinate represents $-\log(P)$. Bars that exceed the blue and red dotted lines indicate pathways with $P < 0.05$ and $P < 0.01$, respectively. T_{3h}, 3 h pre-slaughter transportation; T_{3h}+GAA_{1,200}, 3 h pre-slaughter transportation combined with 1,200 mg/kg guanidine acetic acid supplementation.

unsaturated fatty acids, beta-alanine metabolism, sulfur metabolism, and tyrosine metabolism. The highest-impact pathways between the T_{3h} group and the T_{3h}+GAA_{1,200} group, identified from the discriminated metabolites, were biosynthesis of amino acids, lysine degradation, aminoacyl-tRNA biosynthesis, ABC transporters, glycine, serine and threonine metabolism, purine metabolism, phenylalanine, tyrosine and tryptophan biosynthesis, arginine and proline metabolism, beta-alanine metabolism, cysteine and methionine metabolism, glyoxylate and dicarboxylate metabolism, glutathione metabolism, carbon metabolism, TCA cycle, 2-oxocarboxylic acid metabolism, phosphonate, and phosphinate metabolism.

mRNA Expression of GAMT and CrT in PM and Liver

As shown in Figure 6, there were no differences in mRNA expressions of GAMT in muscle (Figure 6A)

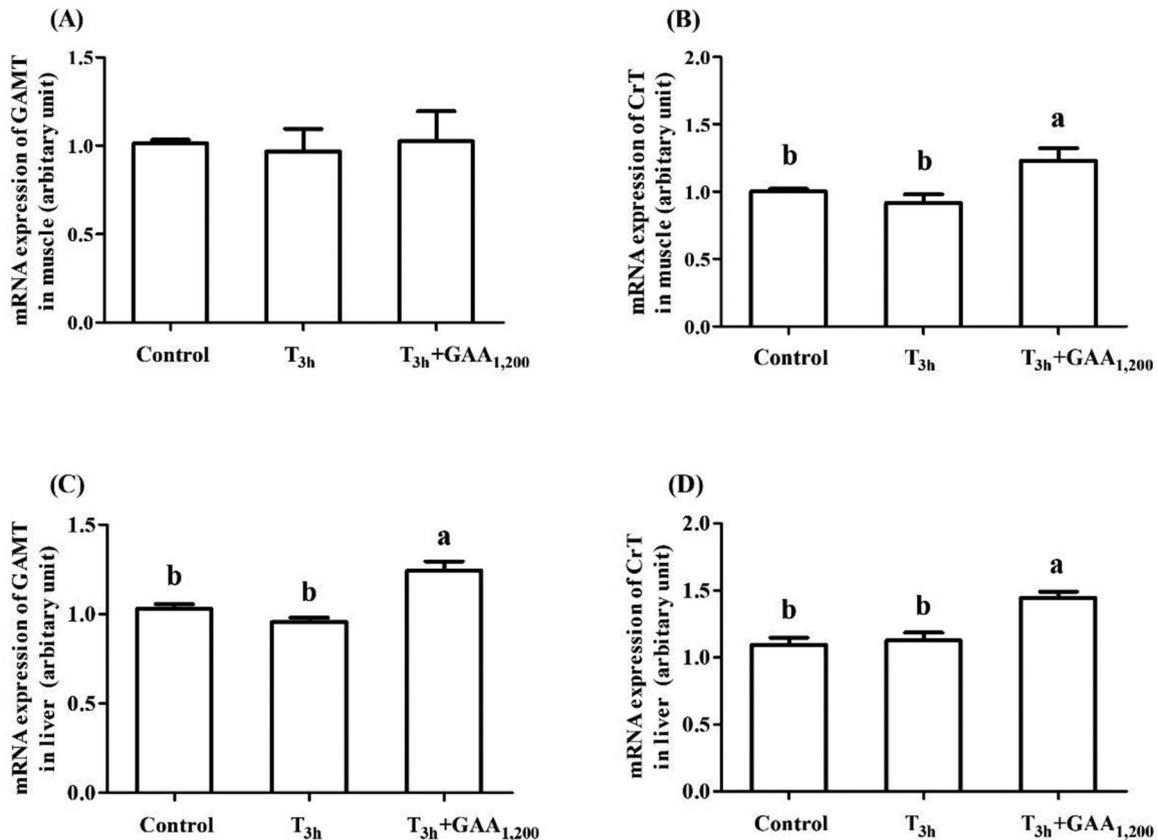


Figure 6. Effects of dietary guanidinoacetic acid (GAA) supplementation on mRNA expression of GAMT and CrT in pectoral muscle (A and B) and liver (C and D) of Qiangdongnan Xiaoxiang chickens subjected to pre-slaughter transport stress ($n = 8$ per treatment). Results are expressed as the mean value and standard deviation. Means within different letters differ significantly ($P < 0.05$). Control, broilers fed the basal diet with no transportation; T_{3h}, broilers fed the basal diet and experienced a 3 h pre-slaughter transportation; T_{3h}+GAA_{1,200}, broilers fed the basal diet supplemented with GAA at 1,200 mg/kg and experienced a 3 h pre-slaughter transportation. Abbreviations: CrT, creatine transporter; GAMT, S-adenosyl-L-methionine: guanidinoacetate N-methyltransferase.

and liver (Figure 6C) of broilers between the control group and the T_{3h} group ($P > 0.05$). Similar results occurred in mRNA expressions of CrT in muscle (Figure 6B) and liver (Figure 6D) of broilers between the control group and the T_{3h} group ($P > 0.05$). Besides, there were also no differences in mRNA expressions of GAMT in muscle of broilers between the T_{3h} group and the T_{3h}+GAA_{1,200} group ($P > 0.05$). However, compared with the T_{3h} group, 1,200 mg/kg GAA supplementation elevated GAMT mRNA expressions of broilers in liver and CrT mRNA expressions in liver and muscle ($P < 0.05$).

DISCUSSION

It has been reported that dietary supplemented with 1,200 mg/kg GAA did not affect ADFI, ADG, or F/G of broilers (Nasiroleslami et al., 2018). Similarly, our previous study also demonstrated that ADFI, ADG, and F/G of broilers were not affected by dietary 1,200 mg/kg GAA supplementation for 14 d (Zhang et al., 2021). Zhang et al. (2019b) suggested that there were no differences in ADG, ADFI, or feed efficiency of broilers fed with diets containing 1,200 mg/kg GAA for 14 d prior to pre-slaughter. In this study, no differences in ADFI,

ADG, or F/G were observed by 1,200 mg/kg GAA supplementation.

The results of our present study demonstrated that 3 h pre-slaughter transport resulted in a higher corticosterone concentration and lower glucose content in plasma, which were considered as the indicators in response to pre-slaughter transport stress (Zhang et al., 2021). Wang et al. (2017) (Wang et al., 2017) suggested that the concentration of corticosterone was elevated by 3 h pre-slaughter transport, indicating that broilers suffered from strong psychological or physiological stress during 3-h transport duration. However, dietary supplemented with 1,200 mg/kg GAA showed lower corticosterone content. In accordance with the results of ours, Zhang et al. (2019b) suggested that GAA supplementation decreased the concentration of corticosterone in plasma of broilers compared with those in 3 h pre-slaughter transport stress group, indicating that GAA supplementation exerted potential effects on alleviating the stress response induced by 3 h pre-slaughter transport.

The anaerobic glycolysis of PM, which only consists of IIb type fibers (fast glycolytic fibers), was accelerated during transportation stress (Wang et al., 2017). Subsequently, lactic acid was accumulated owing to the rapid glycolysis and the pH value of meat was lowered, and then resulted in an inferior meat. Our results showed

that 3 h pre-slaughter transport stress decreased pH_{24h} of postmortem PM, accompanied by the increased L* value, drip loss and cooking loss, which were consistent with the previous reports (Simões et al., 2009; Xing et al., 2016). In contrast, 1,200 mg/kg GAA addition improved meat quality, evidenced as higher pH_{24h} value, lower drip loss, cooking loss, and L* value when compared with broilers subjected to 3 h pre-slaughter transport stress.

Broilers may be exposed to many potential factors pre-slaughter such as catching, crating, feed and water deprivation, transport, and lairage, which could affect animal welfare through exhaustion of their energy stores (Savenije et al., 2002; Vosmerova et al., 2010). ATP, the major high-energy compound in cells, could provide energy for PM through phosphoric acid hydrolysis. In addition, the important energy-buffering Cr and PCr system acted as an energy transport system, which could carry high-energy phosphates from mitochondrial production sites to energy utilization sites and regulate cellular energy homeostasis during periods of high-energy demand or energy supply fluctuations (Wyss and Kadurah-Daouk, 2000; Zhang et al., 2021). The results of our study demonstrated that 3-h pre-slaughter transport stress increased the energy expenditure of PM, as evidenced by the decreased levels of Cr and ATP and the increase in concentration of ADP, AMP, and the ratio of AMP:ATP, which was in accordance with the results of Zhang et al. (2017b). However, broilers in the T_{3h}+GAA_{1,200} group exhibited higher ATP, Cr, and PCr levels and lower ADP, AMP and the ratio of AMP:ATP in PM in comparison with those in the T_{3h} group, indicating GAA could effectively improve the energy metabolism of PM. Similarly, it was reported that GAA supplementation increased the Cr and PCr content of PM while the AMP concentration of PM was decreased (Tossenberger et al., 2016), implying that GAA was an efficient Cr source to increase energy stores in the form of Cr and PCr.

PCA and OPLS-DA analysis were performed to identify the variations in metabolites in PM between the control group and the T_{3h} group, as well as the T_{3h} group and the T_{3h}+GAA_{1,200} group. To the best of our knowledge, this study is the first to identify predictive biomarkers of broilers exposed to pre-slaughter transport stress combined with GAA supplementation. The results of PCA showed that there were significant changes in metabolites between the control group and the T_{3h} group, as well as between the T_{3h} group and the T_{3h}+GAA_{1,200} group. Similar results were also observed in the results of OPLS-DA, where samples from the same treatment group were aggregated into a cluster and completely separated from other groups. Notably, as shown in Table 6, comparing the different metabolites between the control group and the T_{3h} group indicated that abundant substances were involved in amino acid metabolism and oxidative stress, including alanine, glycine, 3-methylhistidine, 1-methylhistidine, and glutathione. Alanine, which was the main glucogenic amino acid and a product of muscle amino acid degradation that

produces glucose via the Cahill cycle, was downregulated in PM of broilers in the T_{3h} group, indicating that more alanine involved in amino acid catabolism as an energy source in a context of lack of carbohydrate energy supply (Beauclercq et al., 2016). In our study, the results showed that glycine, 3-methylhistidine, and 1-methylhistidine were lower in broilers of the T_{3h} group in comparison with those in the control group. It was reported that glycine was involved in the biosynthesis of glutathione and creatine, which play important roles in antioxidant defense and energy production, respectively. Moreover, 3-methylhistidine and 1-methylhistidine recognized markers of the proteolysis of skeletal PM proteins, also have antioxidant properties (Kohen et al., 1988). In previous studies, it was proven that pre-slaughter transport stress disturbed the balance in the oxidant-antioxidant defense system, inducing immunosuppressive effects in some animals (Pan et al., 2018; Zhang et al., 2019a). In accordance with this, the results of our study showed that broilers in the T_{3h} group exhibited lower glutathione levels in PM when compared with those in the control group. But the specific mechanism by which pre-slaughter transport stress affects the oxidant defense system needs further study.

By comparing the metabolites between the T_{3h} group and the T_{3h}+GAA_{1,200} group, it was found that GAA supplementation altered the levels of 48 metabolites, many of which were involved in amino acid metabolism including glycine, serine and threonine metabolism, purine metabolism, phenylalanine, tyrosine and tryptophan biosynthesis, arginine and proline metabolism, β -alanine metabolism, biosynthesis of amino acids, cysteine and methionine metabolism, and citrate cycle (Table 7). The lower concentrations of Cr and PCr in broilers of the T_{3h} group, combined with a higher content of lactic acid, indicated that broilers were in a negative energy balance. Cellular energy requirements could be met by the metabolism of fatty acids and amino acids, except for glucose, through a series of highly integrated chemical reactions under stress conditions to sustain energy demand and maintain muscle mass (Neto et al., 2020). These results suggest that protein may be involved in decomposition for energy (Lu et al., 2018), which could explain the differences in amino acids between the T_{3h} and the T_{3h}+GAA_{1,200} group. In addition, the non-protein amino acid (citrulline) was elevated in the T_{3h} group, possibly owing to its special functions in stress relief and thermoregulation (Monti and Jantos, 2004; Suenaga et al., 2008). Moreover, there were variations in energy metabolism (Cr, PCr, sarcosine, and glycoamine) between the T_{3h} and the T_{3h}+GAA_{1,200}, indicating that exogenous GAA supplementation was helpful in improving energy storage to avoid the aggravated energy expenditure induced by pre-slaughter transport stress.

In order to identify the reasons for the significant differences in Cr and PCr in PM, further analysis of their related genes was conducted using RT-PCR. GAA could be endogenously synthesized from glycine and arginine catalyzed by the enzyme AGAT in the kidney and

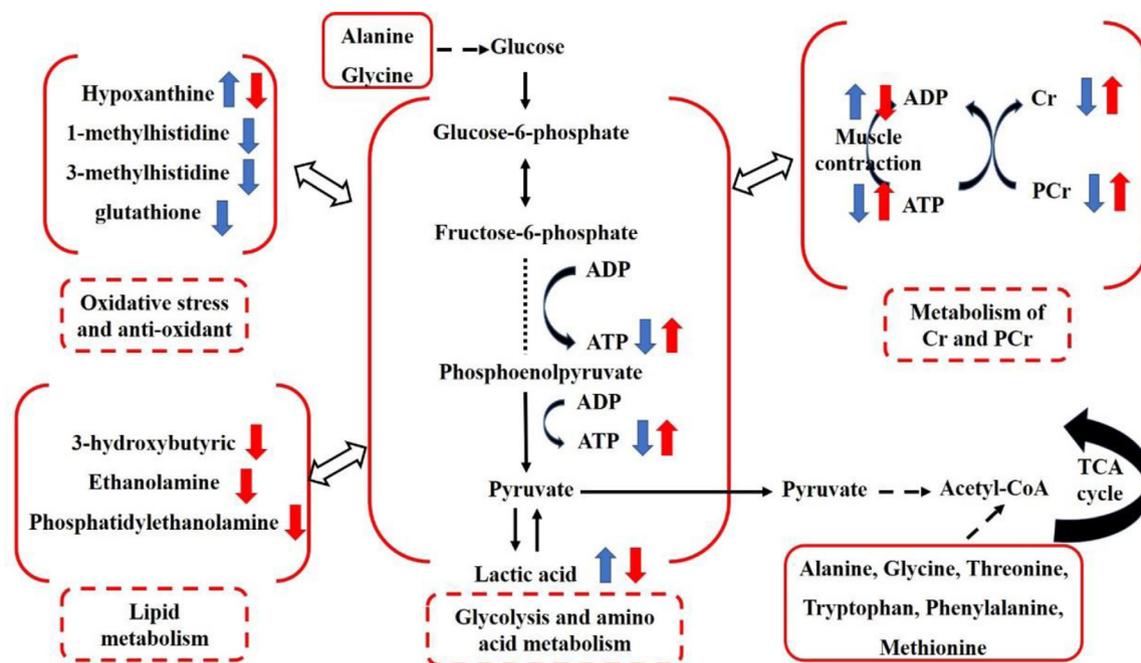


Figure 7. Overview of some important metabolites and major metabolic pathway linked to glycolysis and amino acid metabolism, lipid metabolism, Cr and PCr metabolism and oxidative stress and antioxidant pathway in broilers treated with pre-slaughter transport and GAA supplementation. “↑” and “↓” mean that the metabolites were upregulated and downregulated by pre-slaughter transport stress, respectively; “↑” and “↓” mean that the metabolites were upregulated and down-regulated by 1,200 mg/kg guanidinoacetic acid supplementation, respectively. Abbreviations: ADP, adenosine diphosphate; ATP, adenosine triphosphate; Cr, creatine; PCr, phosphocreatine.

pancreas of normal animals. GAA can be converted to Cr via methylation in the liver with S-adenosylmethionine as the methyl group donor through the action of the enzyme GAMT, and subsequently released into the circulation to creatine-requiring tissue cells via CrT (Christie, 2007). In this study, GAMT mRNA expression in muscle was not affected by 3 h pre-slaughter transport stress or 1,200 mg/kg GAA supplementation, which was consistent with the results of a previous report (Zhang et al., 2019b). In contrast, compared with broilers in T_{3h} group, 1,200 mg/kg GAA supplementation increased GAMT mRNA level in the liver of broilers and CrT mRNA expression in muscle and liver. Consistently, Li et al (2018) demonstrated that GAA supplementation increased GAMT mRNA level in the liver, suggesting that GAA could promote the synthesis of creatine. Besides, the increased mRNA expression of CrT exhibited in broilers of the T_{3h} + GAA_{1,200} group was in accordance with the enhanced Cr and PCr contents in PM in response to GAA supplementation. Consistent with our results, it was demonstrated that GAA supplementation elevated Cr and PCr contents and mRNA expression of the Cr transporter in muscle (Liu et al., 2015), indicating that GAA could be used as a good source of Cr to improve the level of energy sources in PM to resist the negative effects on energy metabolism in broilers induced by pre-slaughter transport stress.

In conclusion, metabolomics approaches to assess the metabolite variations in PM in response to pre-slaughter transport and GAA supplementation showed that there were significant metabolic differences, including carbohydrate metabolism, energy metabolism and oxidative stress metabolism (Figure 7). Furthermore, the present study

also demonstrated that GAA supplementation before slaughter contributed to enhancing the content of high-energy compounds in PM and ameliorating meat quality of broilers induced by pre-slaughter transport stress.

ACKNOWLEDGMENTS

The authors are grateful to Rongjiang Shannong Development Co., Ltd for providing Qiangdongnan Xiaoxiang chickens. The study was supported by National Natural Science Foundation of China (No. 31760674), Zunyi 15851 Talent Project (2050020213#), Characteristic Laboratory of Animal Resources Conservation and Utilization of Chishui River Basin (Qianjiaohe KY [2013]111), the Rural Industrial Revolution Project of Guizhou Province (Zunshi he Rural Industry 201905), Zunshi Chi Shuihe Fund (Zunshi CSHKJ [2019]-01),

DISCLOSURES

The authors declare no competing financial interest.

REFERENCES

- Beauclercq, S., L. Nadal-Desbarats, C. Hennequet-Antier, A. Collin, S. Tesseraud, M. Bourin, E. Le Bihan-Duval, and C. Berri. 2016. Serum and muscle metabolomics for the prediction of ultimate pH, a key factor for chicken-meat quality. *J. Proteome Res.* 15:1168–1178.
- Boerboom, G., T. van Kempen, A. Navarro-Villa, and A. Pérez-Bonilla. 2018. Unraveling the cause of white striping in broilers using metabolomics. *Poult. Sci.* 97:3977–3986.
- Christie, D. L. 2007. Functional insights into the creatine transporter. *Sub-cell Biochem.* 46:99–118.

- Hussain, F., A. Mahmud, S. Mehmood, and M. H. Jaspal. 2020. Effect of broiler crating density and transportation distance on preslaughter losses and physiological response during the winter season in Punjab, Pakistan. *Braz. J. Poult. Sci.* 22:1–10.
- Kohen, R., Y. Yamamoto, K. C. Cundy, and B. N. Ames. 1988. Antioxidant activity of carnosine, homocarnosine, and anserine present in muscle and brain. *Proc. Natl. Acad. Sci. U.S.A.* 85:3175–3179.
- Li, J. L., L. Zhang, Y. N. Fu, Y. J. Li, Y. Jiang, G. H. Zhou, and F. Gao. 2018. Creatine monohydrate and guanidinoacetic acid supplementation affects the growth performance, meat quality, and creatine metabolism of finishing pigs. *J. Agr. Food Chem.* 66:9952–9959.
- Liu, L., M. Ren, Y. Yang, and Z. Chen. 2020. Characterization and phylogenetic analysis of the complete mitochondrial genome in Xiaoxiang chicken (*Gallus gallus domesticus*). *Mitochondrial DNA B.* 5:699–700.
- Liu, Y., J. L. Li, Y. J. Li, T. Gao, L. Zhang, F. Gao, and G. H. Zhou. 2015. Effects of dietary supplementation of guanidinoacetic acid and combination of guanidinoacetic acid and betaine on postmortem glycolysis and meat quality of finishing pigs. *Anim. Feed Sci. Tech.* 205:82–89.
- Livak, K. J., and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. *Methods* 25:402–408.
- Lu, Z., X. F. He, B. B. Ma, J. L. Zhang, Li, Y. Jiang, G. H. Zhou, and F. Gao. 2018. Serum metabolomics study of nutrient metabolic variations in chronic heat-stressed broilers. *Br. J. Nutr.* 119:771–781.
- McBreairty, L. E., J. L. Robinson, K. R. Furlong, J. A. Brunton, and R. F. Bertolo. 2015. Guanidinoacetate is more effective than creatine at enhancing tissue creatine stores while consequently limiting methionine availability in Yucatan miniature pigs. *PLoS One* 10:e0131563.
- Michiels, J., L. Maertens, J. Buyse, A. Lemme, M. Rademacher, N. A. Dierick, and S. De Smet. 2012. Supplementation of guanidinoacetic acid to broiler diets: effects on performance, carcass characteristics, meat quality, and energy metabolism. *Poult. Sci.* 91:402–412.
- Monti, J. M., and H. Jantos. 2004. Effects of L-arginine and SIN-1 on sleep and waking in the rat during both phases of the light/dark cycle. *Life Sci.* 75:2027–2034.
- Nasiroleslami, M., M. Torki, A. A. Saki, and A. R. Abdolmohammadi. 2018. Effects of dietary guanidinoacetic acid and betaine supplementation on performance, blood biochemical parameters and antioxidant status of broilers subjected to cold stress. *J. Appl. Anim. Res.* 46:1016–1022.
- Neto, I. V. d. S., M. M. Carvalho, R. d. C. Marqueti, J. A. Almeida, K. d. S. Oliveira, F. R. Barin, B. Petriz, H. S. S. d. Araújo, O. L. Franco, and J. L. Q. Durigan. 2020. Proteomic changes in skeletal muscle of aged rats in response to resistance training. *Cell Biochem. Funct.* 38:1–10.
- Ostojic, S. M. 2015. Guanidinoacetic acid as a performance-enhancing agent. *Amino Acids* 48:1867–1875.
- Pan, L., X. K. Ma, P. F. Zhao, Q. H. Shang, S. F. Long, Y. Wu, and X. S. Piao. 2018. Forsythia suspensa extract attenuates breast muscle oxidative injury induced by transport stress in broilers. *Poult. Sci.* 97:1554–1563.
- Savenije, B., E. Lambooi, M. Gerritzen, K. Venema, and J. Korf. 2002. Effects of feed deprivation and transport on pre-slaughter blood metabolites, early postmortem muscle metabolites, and meat quality. *Poult. Sci.* 81:699–708.
- Simões, G. S., A. Oba, T. Matsuo, A. Rossa, M. Shimokomaki, and E. I. Ida. 2009. Vehicle thermal microclimate evaluation during Brazilian summer broiler transport and the occurrence of PSE (Pale, Soft, Exudative) meat. *Braz. Arch. Biol. Techn.* 52:195–204.
- Suenaga, R., H. Yamane, S. Tomonaga, M. Asechi, N. Adachi, Y. Tsuneyoshi, I. Kurauchi, H. Sato, D. M. Denbow, and M. Furuse. 2008. Central L-arginine reduced stress responses are mediated by L-ornithine in neonatal chicks. *Amino Acids* 35:107–113.
- Tossenberger, J., M. Rademacher, K. Németh, V. Halas, and A. Lemme. 2016. Digestibility and metabolism of dietary guanidinoacetic acid fed to broilers. *Poult. Sci.* 95:2058–2067.
- Vosmerova, P., J. Chloupek, I. Bedanova, P. Chloupek, K. Kruzikova, J. Blahova, and V. Vecerek. 2010. Changes in selected biochemical indices related to transport of broilers to slaughterhouse under different ambient temperatures. *Poult. Sci.* 89:2719–2725.
- Wallimann, T., M. Tokarska-Schlattner, and U. Schlattner. 2011. The creatine kinase system and pleiotropic effects of creatine. *Amino Acids* 40:1271–1296.
- Wang, X., J. Li, J. Cong, X. Chen, X. Zhu, L. Zhang, F. Gao, and G. Zhou. 2017. Preslaughter transport effect on broiler meat quality and postmortem glycolysis metabolism of muscles with different fiber types. *J. Agr. Food Chem.* 65:10310–10316.
- Wu, Y., Z. Lei, Y. Wang, D. Yin, S. E. Aggrey, Y. Guo, and J. Yuan. 2021. Metabolome and microbiota analysis reveals the conducive effect of BCC-1 and xylan oligosaccharides on broiler chickens. *Front. Microbiol.* 12:683905.
- Wyss, M., and R. Kaddurah-Daouk. 2000. Creatine and creatinine metabolism. *Physiol. Rev.* 80:1107–1213.
- Xia, J., I. V. Snelnikov, B. Han, and D. S. Wishart. 2015. MetaboAnalyst 3.0—making metabolomics more meaningful. *Nucleic Acids. Res.* 43:W251–W257.
- Xing, T., X. Xu, N. Jiang, and S. Deng. 2016. Effect of transportation and pre-slaughter water shower spray with resting on AMP-activated protein kinase, glycolysis and meat quality of broilers during summer. *Anim. Sci. J.* 87:299–307.
- Yalcin, S., and H. C. Guler. 2012. Interaction of transport distance and body weight on preslaughter stress and breast meat quality of broilers. *Br. Poult. Sci.* 53:175–182.
- Zanetti, E., S. Yalçın, C. Guler, and M. Cassandro. 2011. A note on the effect of pre-slaughter transport duration on nutrient composition and fatty acid profile of broiler breast meat. *J. Anim. Feed Sci.* 20:412–419.
- Zhang, C., Z. Y. Geng, K. K. Chen, X. H. Zhao, and C. Wang. 2019a. L-theanine attenuates transport stress-induced impairment of meat quality of broilers through improving muscle antioxidant status. *Poult. Sci.* 98:4648–4655.
- Zhang, L., J. L. Li, X. F. Wang, X. D. Zhu, F. Gao, and G. H. Zhou. 2019b. Attenuating effects of guanidinoacetic acid on preslaughter transport-induced muscle energy expenditure and rapid glycolysis of broilers. *Poult. Sci.* 98:3223–3232.
- Zhang, B. L., N. Liu, Z. He, P. Y. Song, M. L. Hao, Y. X. Xie, J. H. Li, R. J. Liu, and Z. W. Sun. 2021. Guanidino-acetic acid: a scarce substance in biomass that can regulate postmortem meat glycolysis of broilers subjected to pre-slaughter transportation. *Front. Bioeng. Biotech.* 8:631194.
- Zhang, L., X. F. Wang, J. L. Li, X. D. Zhu, F. Gao, and G. H. Zhou. 2017b. Creatine monohydrate enhances energy status and reduces glycolysis via inhibition of AMPK pathway in pectoralis major muscle of transport-stressed broilers. *J. Agr. Food Chem.* 65:6991–6999.
- Zhang, C., L. Wang, X. H. Zhao, X. Y. Chen, L. Yang, and Z. Y. Geng. 2017a. Dietary resveratrol supplementation prevents transport-stress-impaired meat quality of broilers through maintaining muscle energy metabolism and antioxidant status. *Poult. Sci.* 96:2219–2225.
- Zhang, L., H. Y. Yue, H. J. Zhang, L. Xu, S. G. Wu, H. J. Yan, Y. S. Gong, and G. H. Qi. 2009. Transport stress in broilers: I. Blood metabolism, glycolytic potential, and meat quality. *Poult. Sci.* 88:2033–2041.