Serum Metabolomics of Acute Heat-Stressed Broiler Chickens by Gas Chromatography-Mass Spectrometry Postprint

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Date: 2018-12-24T00:00:00+00:00

Abstract

This study aimed to utilize gas chromatography-mass spectrometry (GC-MS) technology to analyze serum metabolomic changes in broiler chickens under acute heat stress, identify differential metabolites and differential metabolic pathways, and reveal the underlying mechanisms of nutrient metabolic pathway alterations in broiler chickens under acute heat stress at the metabolomic level. The experiment selected 20 Arbor Acres (AA) broiler chickens at 35 days of age, which were randomly and equally divided into 2 groups, with 5 replicates per group and 6 chickens per replicate. The control group was raised at standard temperature according to conventional feeding standards, while the heat stress group was raised at (32±1) °C, both for 12 hours. Blood (2 mL) was collected from the brachial vein, serum was separated, and after derivatization treatment, GC-MS technology was used to obtain serum metabolic profiles of the two groups of broiler chickens. The collected data were subjected to principal component analysis and partial least squares discriminant analysis of metabolites to identify differential metabolites and perform enrichment analysis of differential metabolic pathways. The results showed that: using GC-MS technology, a total of 144 metabolites were detected in broiler serum, and 30 differential metabolites were screened out [variable importance in projection (VIP) > 1, $P < 0.05$]. Among them, 14 metabolites including fumaric acid, hydroxybutyric acid, and alanine were up-regulated [fold change (FC) > 1], while 16 metabolites including oxalic acid, malic acid, and dihydroxyacetone were down-regulated (FC < 1). Metabolic pathway enrichment analysis revealed that metabolic pathways such as the tricarboxylic acid cycle, galactose metabolism, propionate metabolism, and fatty acid biosynthesis were significantly altered ($P < 0.05$ or $P < 0.01$). These results indicate that serum metabolites and metabolic pathways were significantly altered in broiler chickens under acute heat stress. The identified 30 differential metabolites and 7 differential metabolic pathways may serve as biomarkers for elucidating the pathogenesis of acute heat stress.

Full Text

Preamble

Metabonomics Study on Serum Substance Metabolomics in Acutely Heat-Stressed Broilers Using Gas Chromatography-Mass Spectrometry Technique

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Abstract: This study aimed to investigate changes in serum substance metabolomics of acutely heat-stressed broilers using gas chromatography-mass spectrometry (GC-MS) technology, identify differential metabolites and metabolic pathways, and reveal the mechanisms underlying nutrient metabolic pathway alterations in broilers during acute heat stress at the metabolomic level. Twenty 35-day-old Arbor Acres (AA) broilers were randomly divided into two groups with five replicates per group and six broilers per replicate. The control group was maintained at standard feeding temperature, while the heat stress group was housed at (32±1) °C for 12 hours. Blood samples (2 mL) were collected from the wing vein, and serum was separated and derivatized. GC-MS technology was employed to obtain serum metabolic profiles of both groups. Principal component analysis and partial least squares discriminant analysis were performed on the collected data to identify differential metabolites and conduct metabolic pathway enrichment analysis. The results showed that 144 metabolites were detected in broiler serum using GC-MS, and 30 differential metabolites were screened [variable importance in projection (VIP)>1, $P<0.05$]. Among these, 14 metabolites including fumaric acid, hydroxybutyric acid, and alanine were upregulated [fold change (FC)>1], while 16 metabolites including oxalic acid, malic acid, and dihydroxyacetone were downregulated (FC<1). Metabolic pathway enrichment analysis revealed significant alterations in tricarboxylic acid cycle, galactose metabolism, propionate metabolism, and fatty acid biosynthesis pathways ($P<0.05$ or $P<0.01$).

These findings demonstrate that serum metabolites and metabolic pathways undergo significant changes during acute heat stress in broilers. The identified 30 differential metabolites and 7 differential metabolic pathways may serve as biomarkers for elucidating the pathogenesis of acute heat stress.

Keywords: broilers; heat stress; metabonomics; tricarboxylic acid cycle; fatty acid biosynthesis

Research indicates that broilers exhibit significant heat stress responses when environmental temperature reaches 32 °C [**?**]. To adapt to high-temperature environments, broilers actively alter the metabolism of various substances to maintain thermal balance. The severity of heat stress damage is closely related to changes in nutrient absorption and metabolism induced by heat stress [**?**].

Previous studies investigating changes in partial serum biochemical indices during heat stress in broilers have preliminarily confirmed significant alterations in nutrient absorption and metabolism [**?**, **?**]. However, these data cannot systematically and comprehensively reveal the overall status of important nutrient absorption and metabolism, and the differential metabolic markers and metabolic pathway changes during heat stress remain unclear, hindering approaches to alleviate heat stress damage through regulation of nutrient absorption and metabolism.

Metabolomics research can more directly, accurately, and systematically reflect the total metabolites in organisms under various life-sustaining states using high-throughput analytical techniques. Identifying specific biomarkers during disease development through metabolomics can provide a basis for early diagnosis and targeted therapeutic measures [**?**, **?**]. Detecting the complete profile of small molecules in serum during heat stress can help further reveal the mechanisms of heat stress damage in broilers, yet studies analyzing all serum metabolite changes using metabolomic approaches are lacking. Therefore, this study employed gas chromatography-mass spectrometry (GC-MS) technology combined with pattern recognition methods to investigate serum metabolomic changes in broilers during acute heat stress, identify differential metabolites and metabolic pathway alterations, further elucidate the mechanisms of heat stress in broilers, and provide new insights for early diagnosis and prevention.

1.1 Experimental Design

The chicken house was cleaned and disinfected one week before the experiment, with temperature and humidity controlled using electronic heaters and humidifiers. Commercial Arbor Acres (AA) broilers were purchased and adaptively raised to 28 days of age according to feeding standards. Sixty healthy AA broilers were randomly divided into two groups: a standard feeding group (control) and a heat environment group (heat stress), with five replicates per group and six broilers per replicate, housed in the same cage. Adaptive feeding continued for seven days until broilers reached 35 days of age, when the formal experiment began. The control group was maintained at standard feeding temperature, while the heat stress group was exposed to (32±1) °C for 12 hours. Relative humidity in both houses was controlled at (60±5)%. The basal diet composition and nutrient levels are shown in Table 1 .

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

Ingredients	Content	Nutrient levels	Content
Corn		Metabolic energy (ME) (MJ/kg)	
Soybean meal		Crude protein (CP)	
Fish powder		Total phosphorus (TP)	
CaHPO4		Lysine (Lys)	

Ingredients	Content	Nutrient levels	Content
NaCl		Methionine + Cysteine (Met+Cys)	
Premix			
Total			

1) The premix provided the following per kilogram of diet: Mn 66 mg, Zn 44 mg, Cu 9 mg, Fe 50 mg, I 0.4 mg, VA 7,000 IU, VD3 875 IU, VE 20 mg, VK3 1 mg, VB1 2 mg, VB2 4.5 mg, D-calcium pantothenate 12 mg, nicotinic acid 50 mg, VB6 2.5 mg, VB12 0.6 mg.

2) Nutrient levels were calculated values.

1.2 Sample Preparation

After 12 hours of treatment, two broilers were randomly selected from each replicate (cage), with 10 broilers per group selected for the experiment. Non-anticoagulated blood (2 mL) was collected from the unilateral wing vein. Blood samples were placed in a 4 °C refrigerator for 12 hours, then incubated in a 37 °C water bath for 20 minutes, and centrifuged at 4 °C and 3,000 r/min for 10 minutes to collect serum for subsequent analysis.

1.3 Sample Pretreatment

Serum and L-2-chlorophenylalanine (0.3 mg/mL) were mixed at a 1:5 ratio in a centrifuge tube and vortexed for 10 seconds. A methanol and acetonitrile solution (2:1 volume ratio, 150 L) was added to precipitate proteins, followed by standing at -20 °C for 10 minutes. After ultrasonic-assisted extraction, samples were cooled again at -20 °C for 10 minutes and centrifuged at 15,000 r/min for 10 minutes. The supernatant (150 L) was evaporated to dryness, then 80 L of 15 mg/mL methoxyamine hydrochloride pyridine solution was added for oximation. Subsequently, 80 L of N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) derivatization reagent containing 1% trimethylchlorosilane (TMCS) and 20 L of n-hexane were added, and the mixture was reacted at 70 °C for 60 minutes. Samples were removed and placed at room temperature for 30 minutes before metabolomic analysis by GC-MS.

1.4 GC-MS Analysis

A gas chromatography time-of-flight mass spectrometer (7890A-5975C, Agilent, USA) was used for data acquisition. Prepared samples were injected into the GC-MS system in splitless mode, separated by a DB-5MS capillary column, and detected by mass spectrometry. High-purity helium was used as carrier gas at a flow rate of 1.0 mL/min. The temperature program was: 50–125 °C at 15 °C/min; 125–210 °C at 5 °C/min; 210–270 °C at 10 °C/min; 270–305 °C at 20 °C/min; held at 305 °C for 5 minutes. Injector temperature, electron impact

ionization source temperature, and voltage were set at 260 °C, 230 °C, and 70 V, respectively. Mass scan range (m/z) was 50–600, with signal acquisition starting at 20 spectra/s after 5 minutes.

1.5 Quality Control Samples

Quality control samples were prepared by thoroughly mixing serum extracts from both AA broiler groups. QC samples were processed and detected using the same method. During instrumental analysis, one QC sample was inserted after every five analytical samples to verify the accuracy and reproducibility of the analytical process.

1.6 Data Analysis

Raw GC-MS data were preprocessed using ChromaTOF software (v 4.34, LECO, USA) to export three-dimensional data matrices in CSV format containing sample information, retention time-mass-to-charge ratio, and mass spectral response intensity. Internal standard peaks and false-positive peaks (including noise, column bleed, and derivatization reagent peaks) were removed, followed by deredundancy and peak merging, yielding 144 metabolites from the analytical samples. The normalized response intensity data matrix of mass spectral peaks was imported into SIMCA-P+14.0 software for principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) to analyze the overall distribution among samples, analytical stability, and overall differences in metabolic profiles between groups. In PLS-DA analysis, variables with variable importance in projection (VIP) greater than 1 were considered differential variables. Seven-fold cross-validation and 200-response permutation testing were employed to prevent model overfitting.

1.7 Metabolite Identification

Differential metabolites between control and heat stress groups were screened by combining PLS-DA multivariate analysis and t-test univariate analysis (VIP>1, P<0.05). For metabolite identification, the GC-MS workstation software automatically compared the mass-to-charge ratio and abundance of characteristic ion fragment spectra for each detected substance against the NIST database and Feihn metabolomics database, with standard substances required to have matching degrees exceeding 70%.

1.8 Metabolic Pathway Enrichment Analysis

The MBRole database (http://csbg.cnb.csic.es/mbrole/) was used for ID conversion to obtain KEGG database (http://www.genome.jp/kegg/pathway.html) substance IDs for differential metabolites. Combined pathway analysis and enrichment functions from both databases were used to identify significantly different metabolic pathways between control and heat stress groups, and metabolic

pathway maps and enrichment analysis histograms for heat-stressed broilers were downloaded.

2 Results

2.1 GC-MS Spectra of Serum Samples from Two Groups

Figure 1 [Figure 1: see original paper] shows representative total ion chromatograms of serum samples from control and heat stress groups. The figure demonstrates strong signals for serum metabolites during the experiment, with peak retention times and intensities meeting experimental requirements. Comparison of serum mass spectra with the NIST database identified 144 metabolites with matching degrees exceeding 70%. Multivariate and univariate statistical analyses were combined to characterize metabolic differences between heat stress and control groups.

Figure 1 Total ion chromatograms (TICs) of control group (A) and heat-stressed group (B) by GC-MS analysis

2.2.1 PCA Analysis

Figure 2 [Figure 2: see original paper] presents PCA analysis results for serum metabolites from both groups. Valid samples fell within the 95% confidence interval. PCA analysis showed high explanatory power ($R^2X=0.514>0.5$), confirming model reliability [**?**]. The control and heat stress groups were distributed in different quadrants, indicating genuine inter-group differences and authentic variability among experimental groups.

Figure 2 PCA score scatter plots of serum samples

2.2.2 PLS-DA Analysis

To eliminate noise information unrelated to classification, a PLS-DA model was established based on the PCA model ($R^2X=0.808$, $R^2Y=0.936$, $Q^2=0.738$) to further analyze serum metabolic pattern changes between control and heat stress groups. $Q^2=0.738>0.5$ indicated good discriminant ability [**?**]. The PLS-DA model score plot (Figure 3 [Figure 3: see original paper]) showed clear separation of serum metabolites between the two groups along principal component axes, confirming genuine differences. Model robustness was examined using 200 permutations (Figure 4 [Figure 4: see original paper]), with $R^2=0.771>0.5$ and $Q^2=-0.314<0$, demonstrating a robust and reliable model.

Figure 3 PLS-DA score scatter plots of serum samples

Figure 4 Validation plots of PLS-DA with 200 permutation

2.3 Differential Metabolite Identification

Following PLS-DA analysis and applying criteria of VIP>1 and t-test P<0.05, metabolite peak areas were normalized and relatively quantified, yielding 30 differential metabolites (VIP>1, P<0.05). Among these, 14 metabolites including fumaric acid, 3-hydroxybutyric acid, and alanine were upregulated (fold change FC>1), while 16 metabolites including oxalic acid, malic acid, and dihydroxyacetone were downregulated (FC<1) (Table 2).

Table 2 Identification of significantly different metabolites of control group and heat-stressed group

Metabolites	RT/min	VIP	P-value
Fumaric acid			<0.001
3-hydroxybutyric acid			<0.001
N-methyl-DL-alanine			<0.001
Malic acid			<0.001
2-hydroxybutanoic acid			<0.001
Oleic acid			<0.001
2-butyne-1,4-diol			<0.001
Alanine			<0.001
cis-1,2-dihydronaphthalene-1,2-diol			<0.001
Dihydroxyacetone			<0.001
2-amino-2-methylpropane-1,3-diol			<0.001
Sucrose			<0.001
Oxalic acid			<0.001
Malonamide			<0.001
2-deoxyerythritol			<0.001
Inosine			<0.001
N-methylaniline			<0.001
Dihydrosphingosine			<0.001
Sarcosine			<0.001
Palmitoleic acid			<0.001
Oxamic acid			<0.001
Acetanilide			<0.001
Nicotinoylglycine			<0.001
Tetracosane			<0.001
Beta-mannosylglycerate			<0.001
2-deoxy-glucose			<0.001
Galactinol			<0.001
Threo-beta-hydroxyaspartate			<0.001
Arachidonic acid			<0.001
4-hydroxybenzyl cyanide			<0.001

FC values greater than 1 mean that metabolite in control group was more than that in heat-stressed group. P-values were obtained by t-test.

2.4 Metabolic Pathway Enrichment Analysis

Metabolic pathway enrichment analysis results are shown in Figure 5 [Figure 5: see original paper]. Heat stress most significantly affected the tricarboxylic acid cycle pathway ($P<0.01$), followed by propionate metabolism ($P<0.01$). Additionally, enrichment analysis revealed significant changes in galactose metabolism, arginine and proline metabolism, glyoxylate and dicarboxylate metabolism, fatty acid biosynthesis, and unsaturated fatty acid biosynthesis pathways during acute heat stress ($P<0.05$).

Figure 5 Metabolic pathway enrichment analysis

$P<0.01$, $P<0.05$. 1: TCA cycle; 2: propanoate metabolism; 3: galactose metabolism; 4: glyoxylate and dicarboxylate metabolism; 5: fatty acid biosynthesis; 6: biosynthesis of unsaturated fatty acids; 7: arginine and proline metabolism.

3 Discussion

During heat stress, organisms alter nutrient absorption and metabolism to adapt to high environmental temperatures. Studies have shown significant changes in gene transcription and protein expression in various tissues during heat stress [**?**, **?**], but how these changes ultimately lead to metabolic pathway and final metabolite alterations remains unclear. This study identified 144 metabolites in broiler serum using GC-MS, including 30 differential metabolites. Metabolic pathway enrichment analysis revealed the most significant changes in the tricarboxylic acid cycle pathway, with propionate metabolism, galactose metabolism, and fatty acid biosynthesis pathways also significantly altered.

3.1 Abnormal Energy Metabolism

During heat stress, broilers mobilize energy reserves to adapt to environmental conditions. Hu et al. [**?**] demonstrated that heat stress exerts anti-heat stress effects by regulating cellular energy metabolism such as altering AMP-activated protein kinase (AMPK) activity. Additionally, heat stress affects mitochondrial function, and lipid peroxidation products can inhibit enzymes in the tricarboxylic acid cycle and respiratory chain complexes [**?**, **?**]. Our metabolic pathway enrichment analysis revealed the most significant changes in the tricarboxylic acid cycle pathway, suggesting that heat stress may significantly alter the activity of some enzymes in this cycle, preventing normal metabolic participation of intermediate products like fumaric acid and causing significant changes in their content. Meanwhile, significantly reduced serum malic acid content suggests that heat stress particularly affects the conversion step from fumaric acid to malic acid in the tricarboxylic acid cycle. Therefore, adding intermediate metabolites of the tricarboxylic acid cycle to the diet may improve cycle efficiency and enhance heat stress resistance.

Heat stress increases energy consumption and metabolic rate while downregu-

lating fructose and mannose metabolism [?, ?]. Our results showed significantly reduced levels of glucose metabolism-related metabolites such as sucrose, erythritol, and beta-mannose in heat-stressed broilers, indicating abnormal energy metabolism and accelerated carbohydrate breakdown to provide more energy for stress resistance. D-ribose is a precursor of 5-phosphoribosyl and other monosaccharide metabolites, while 5-phosphoribosyl is a raw material for RNA and DNA synthesis. Significantly elevated serum D-ribose in heat-stressed broilers suggests that reduced DNA synthesis during heat stress may be unrelated to D-ribose content but rather due to loss of control in the D-ribose-to-DNA synthesis process [?]. Additionally, D-ribose participates in adenylate synthesis and regeneration (e.g., ATP) to maintain energy balance in cardiac and skeletal muscle. This finding differs from Gu et al. [?], possibly due to differences in heat stress duration. Furthermore, metabolic creatine in muscle can increase muscle glycogen content, thereby improving heat dissipation capacity in heat-stressed broilers [?]. Further KEGG pathway analysis indicated significant alterations in the tricarboxylic acid cycle and abnormal glyoxylate and dicarboxylate metabolism during heat stress, which may relate to citrate and succinate from the glyoxylate pathway acting as catalysts in the biological oxidation respiratory chain, while dicarboxylate transporters transport citrate and succinate into cells for energy synthesis, thereby regulating energy metabolism [?].

3.2 Abnormal Lipid Metabolism

High-temperature environments cause significant changes in basal metabolic rate and increased abdominal fat deposition as an additional energy storage form [?, ?]. Our study found significant differences in multiple lipid metabolism-related indicators including hydroxybutyric acid, oleic acid, and palmitoleic acid in heat-stressed broilers, indicating lipid metabolism disorder consistent with Xiong et al. [?]. Additionally, reduced feed intake during heat stress causes energy deficiency, prompting mobilization of peripheral adipose tissue for energy supply. When generated fatty acids enter the liver and undergo -oxidation to produce large amounts of ketone bodies that cannot be fully utilized by extrahepatic tissues, hyperketonemia occurs [?]. Our results showed significantly elevated hydroxybutyric acid in heat-stressed broilers, indicating that massive fatty acid mobilization and hepatic ketone body synthesis exceeded extrahepatic utilization capacity, leading to significantly increased ketone bodies [?].

Oleic acid, palmitoleic acid, and arachidonic acid are important forms of unsaturated fatty acids in animals. Oleic acid improves energy metabolism efficiency and regulates lipid catabolism and deposition by modulating lipid metabolism-related enzyme activities [?, ?]. Palmitoleic acid regulates glucose metabolism and lipid synthesis, particularly triglycerides, while also modulating lipase activity [?]. Arachidonic acid regulates lipid metabolism both directly as a phospholipid-bound structure and indirectly through leukotrienes, thromboxane A2, and prostaglandin E2 [?]. Significantly increased serum unsaturated

fatty acids in heat-stressed broilers may represent a transient elevation to meet energy metabolism demands and a transport form for directional fat deposition. These results suggest that dietary supplementation with unsaturated acids promoting fat metabolism may help reduce lipid metabolism disorders during heat stress.

Comprehensive analysis of these changes through KEGG pathway analysis revealed significant alterations in both saturated and unsaturated fatty acid synthesis pathways in heat-stressed broilers. Therefore, elucidating the mechanisms of lipid metabolism pathway changes, particularly saturated and unsaturated fatty acid synthesis pathways, represents another important research topic in acute heat stress metabolism.

3.3 Abnormal Amino Acid Metabolism

Carbon skeletons and ammonia are the final forms of amino acid catabolism in vivo. Carbon skeletons can participate in glucose and lipid metabolism, while ammonia is excreted as nitrogenous waste. Our results showed significantly increased alanine content in heat-stressed broilers, presumably related to its involvement in energy metabolism. The alanine-glucose cycle is an important pathway for its energy metabolic function [**?**]. During heat stress, broilers adjust energy metabolism to adapt to high temperatures. As an energy provider, glucose consumption generates large amounts of pyruvate, requiring alanine metabolism participation to maintain continuous energy supply [**?**]. Additionally, arginine and proline can be converted to glutamine, which is deaminated by alanine aminotransferase to -ketoglutarate, an important tricarboxylic acid cycle intermediate providing energy. In this process, alanine is rapidly converted to pyruvate through deamination, promoting the tricarboxylic acid cycle [**?**]. Serum aspartic acid content showed a significant decreasing trend in our experiment. Heat stress affects the circulatory system, and large amounts of aspartic acid are consumed to transport potassium and magnesium ions to cardiac muscle, enhancing contractility, reducing oxygen consumption, and mitigating heat stress damage to the heart. Moreover, heat-stressed broilers require large glucose consumption to combat heat stress damage, and glucogenic amino acids like aspartic acid generate glucose through gluconeogenesis to alleviate glucose deficiency [**?**]. Analysis of amino acid metabolic characteristics during heat stress suggests that dietary supplementation with glucogenic amino acids or those promoting energy absorption may help prevent and control heat stress.

Furthermore, KEGG database analysis revealed significant changes not only in propionate metabolism (KEGG ID: gga00640) but also in arginine and proline metabolism during heat stress. Enhanced protein catabolism during heat stress requires timely ammonia excretion to prevent toxicity, with arginine serving as an important intermediate metabolite in this process. Proline is an important osmotic pressure regulator, and its metabolic pathway changes may relate to altered internal osmotic pressure caused by dehydration during heat stress. Additionally, arginine and proline synthesis and metabolism are closely related to

intestinal function, and their metabolic pathway abnormalities may significantly impact the gut during acute heat stress. Future research strengthening the effects of arginine and proline on intestinal function in heat-stressed broilers may provide new insights into the mechanisms and prevention of acute heat stress.

4 Conclusion

1. This study employed GC-MS-based metabolomics to investigate changes in small molecule metabolites in heat-stressed broiler serum, identifying 30 differential metabolites (14 upregulated and 16 downregulated), demonstrating significant changes in nutrient metabolism during heat stress adaptation.
2. Metabolic pathway enrichment analysis revealed significant alterations in tricarboxylic acid cycle, fatty acid biosynthesis, and arginine and proline metabolism pathways.

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Note: Figure translations are in progress. See original paper for figures.