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ABSTRACT To study the effects of polyphenolic extract from involucres of Castanea mollissima Blume (PICB), a novel approach using gene expression by real time polymerase chain reaction (real-time PCR) coupled with metabolomic profiling technique was established to explain the mechanism of PICB on heatstressed broiler chicks. Four thousand 28-day-old male Arbor Acres (AA) broilers were randomly assigned to 5 groups (4 replicates / group, 20 chicks / replicate), in which group 1 was normal control group fed with basic ration; groups 2, 3, 4, and 5 were fed with the basic ration with a supplementation of 0.2% Vitamin C (VC), or 0.2%, 0.3%, or 0.4% of PICB respectively. After 1 wk of adaptation, heat stress was applied for 7 consecutive days. On d 3 and d 7 of heat stress, the chicks were sacrificed and sampled. The mRNA expression of heat stress protein 70 (HSP70), glutathione peroxidase (GSH-Px), ornithine decarboxylase (ODC), epidermal growth factor (EGF) and epidermal growth factor receptor (EGFR) were detected by real-time PCR using samples from jejunum mucosa. The serum and jejunum mucosa metabolomic profiles of PICB group showing best antioxidative effects and control group at d 3 were studied using the method of the gas chromatography – time of flight mass spectrometry (GT-TOF-MS), followed by principal component analysis and partial least squares-discriminate analysis. Potential biomarkers were found using Student's t-test. The results showed mRNA expressions of HSP70, GSH-Px, ODC, EGF, and EGFR were altered by the supplementation of PICB. PICB exhibited antioxidative and growth promoting effects, and 0.3% PICB supplementation level exhibited the best. Three metabolites in the serum and 5 in the jejunum mucosa were identified as potential biomarkers. They were considered to be in accordance with antioxidative and growth promoting effects of PICB, which involved in the energy metabolism (sorbitol, palmitic acid), carbohydrate metabolism, amino acids metabolism (serine, L-ornithine), glutathione metabolism (glutamate, L-ornithine), GnRH signaling pathway (inositol), etc. These findings provided novel insights into our understanding of molecular mechanism of PICB effects on heat-stressed chicks.

Key words: mRNA expression, Metabolomic profiling, Heat stress, Castanea mollissima Blume involucre polyphenol, Broiler

INTRODUCTION

Polyphenols are the fourth most abundant component in plant tissues next to cellulose, hemicellulose, and lignin. Due to their specific structures, polyphenols have anti-microbial, anti-parasitic and anti-oxidative activities, which could improve the animal performance (Jouany and Morgavi, 2007). The ethanol extract of the involucres of Castanea mollissima Blume were mainly polyphenols and were reported to have strong radicalscavenging and antioxidant activities (Shi et al., 2013 and Li et al., 2015). Castanea sativa Mill. wood extract were able to reduce the malondialdehyde (MDA) content in rabbits, while increasing total superoxide dismutase (T-SOD), total antioxidant capacity (TAOC) and glutathione peroxidase (GSH-Px)

activities under high ambient temperature (Liu et al., 2011). Our previous study reported the polyphenols from the involucres of Castanea mollissima Blume (PICB) had the similar influences on the antioxidative parameters and improves growth performance of heatstressed broiler chicks (Dong et al., 2015). However, no molecular mechanism was studies on the effects of polyphenols. Heat stress induced by high temperature negatively affects a variety of traits such as nutrient digestion and absorption, serum biochemical parameters, metabolism, and consequently decrease growth performance and production of broiler chicks, even leading to death (Luo et al., 2014). Previous studies have demonstrated that high temperature could alter gene expressions and metabolites of plasma (Sun et al., 2015). Heat stress can cause the body to produce excess radical oxygen species, which could affect animal antioxidant status and lipid peroxidation levels (Gu et al., 2008), and the supplementation of polyphenols could mitigate these alterations (Liu et al., 2011 and Dong et al., 2015). Metabolomics is a new discipline in systems biology with "holistic" philosophy. In recent years, the emerging metabolomics held great promise for the discovery of the biochemical alteration and related pathways (Nicholson and Lindon, 2008). Principal component analysis (PCA) and partial least squares-discriminate analysis (PLS-DA) are indispensable pattern recognition methods in metabolomics research. PCA is an unsupervised multivariable statistical method. It is initially carried out to investigate whether two groups can be separated and to find out their metabolic distinction. PLS-DA is a supervised multivariable statistical method. It is used to sharpen an already established (weak) separation between groups of observations plotted in PCA (Liu et al., 2013). To investigate the mechanism of the effects of PICB on the growth and anti-oxidative capacity of heatstressed broilers, the aims of this study were to analyze some growth and anti-oxidative related gene expressions of jejunum mucosa and metabolomic profiling of serum and jejunum mucosa in order to clarify the molecular mechanism of PICB functions.

MATERIALS AND METHODS

Chemicals

The involucres of Castanea mollissima Blume were collected from Huairou Chestnut Station. PICB was extracted according to the method described by Shi et al. (2013) and the polyphenols content was about 52.5%. PICB also included non-tannins, crude fiber, ash, and water (Dong et al., 2015; Schiavone et al., 2008) . All chemicals and reagents were of analytical grade and were obtained from commercial sources. All water was treated in a water purification system. Vitamin C (VC, ascorbic acid) was obtained from Sigma Chemical Co. (St. Louis, MO). The primers were designed and synthesized by Shanghai Sangong Biological Engineering & Technology Services Co. Ltd. (Shanghai, China). Ribitol, methoxyamine hydrochloride, pyridine, N-methyN-(trimethylsilyl) trifluoroacetamide (MSTFA), and chlorotrimethylsilane (TMCS) were purchased from Sigma. Methanol (MeOH) and chloroform (CHCL3) were obtained from the Beijing Chemical Agent Company (HPLC grade, Beijing, China). Ribitol was prepared with water to 0.2 mg/mL as an internal standard (IS). Methoxyamine hydrochloride was dissolved in pyridine to 20 mg/mL. TMCS was dissolved in MSTFA to 1% (v/v). High purity deionized water with 18.2 MΩ/cm was employed. All of these work solutions were stored at 4 to 5 ◦C. An Agilent 6890 N gas chromatograph (Palo Alto, CA) with a DB-5MS capillary column (30 m × 0.25 mm ID × 0.25 µm thickness) was coupled with a Waters Micromass GCT mass spectrometer (Milford, MA). An Eppendorf 5415D centrifuge was purchased from Eppendorf (Eppendorf, Hamburg, Germany). A ZHWY100B shaker was bought from Dibai Experimental Equipment Co Ltd. (Shanghai, China). A DSHZ-300A thermostat water rocker was purchased from Taicang Experimental Equipment Company (Jiangsu, China). ABF-2000 M nitrogen dryer was purchased from Bafang Tech Co. Ltd. (Beijing, China).

Ethical Approval

The study was conducted in accordance with the Regulations of Experimental Animal Administration issued by the State Committee of Science and Technology of People's Republic of China. All experimental

protocols were approved by the Committee for the Care and Use of Experimental Animals, Beijing University of Agriculture.

Experimental Design and Feeds Preparation

The trials were carried out at the experimental base of the State Key Laboratory of Beijing Institute of Animal Husbandry and Veterinary Medicine, Chinese Academy Agricultural Science located in Changping District of Beijing. The group 1 was fed with basal diet as control group (Table 1). Group 2 was fed with the basal diet with a supplementation of VC (0.2% of diet) (Insurk et al., 2014), which is most widely used in practical feeding, as the positive control group; groups 3, 4, and 5 were fed with the basal diet with a supplementation of 0.2%, 0.3%, and 0.4% of PICB, respectively. VC and PICB were mixed with 1 kg of basal diet at first and successively mixed into an appropriate quantity of basal diet to obtain the prefixed inclusion level. The chicks were managed according to Arbor Acres (AA) broiler feeding and immunization procedure. Broilers were fed and watered ad libitum.

Birds Husbandry

Four thousand 28-day-old male AA broilers (1245.87 ± 22.19 g) were randomly assigned to 5 groups described above (4 replicates/group, 20 chicks/replicate). The birds management was reported in our previous paper (Dong et al., 2015). After one week adaptation (temperature 24 ± 0.5 ◦C, relative humidity 65 ± 5%), heat stress was applied for 7 consecutive days for all groups (regime: 9:00–17:00 temperature 34 ± 0.5 ◦C, relative humidity 85 ± 5%;17:00–9:00 temperature 24 ± 0.5 ◦C, relative humidity 65 ± 5%). The health of the animals was monitored daily and involuntary mortality was recorded. In order to avoid excessive suffering, a humane endpoint was pre-fixed as chicks' breathing rate reaching 100 breaths per minute (approximately 4 times normal rate). Chicks were visual observed for breathing rate (2 times/d per 10 min, respectively, at 10:00 and 16:00) counting the chest cavity movement and respiratory behavior during the heat-stressed week. The chick would be treated with euthanasia when its breathing rate reached the humane endpoint. The growth performance of chicks was reported by Dong et al. (2015). On d 3 and d 7 of heat stress, 8 chicks per group (2 per replicate) were randomly chosen and fasted for 12 h with ad libitum access to water. Blood samples were collected by radial venipuncture (wing vein) using a syringe with a 25-gauge needle containing 100 μL 0.1 M ethylenediaminetetraacetic acid (EDTA; anticoagulant) and used for metabolomic profiling analyses. Then the chicks were killed by intravenous sodium pentobarbital injection, and dissected to collect the jejunum mucosa for mRNA expression analysis and metabolomic profiling analysis.

Gene Expressions of Jejunum Mucosa

RNA and cDNA Preparation

Total RNA was isolated from each of the 40 chick jejunum mucosa samples from the 5 groups (8 samples per group) with Trizol (Invitrogen, Carlsbad, CA), according to the manufacturer's protocol. DNase I (Ambion, Austin, TX) digestion was carried out after RNA isolation. RNA concentration and purity were determined by measuring the absorbance at 260 nm and calculating the A260/A280 ratio using a NanoDrop ND-2000 spectrophotometer (SmartSpec plus, BIO-RAD). The ratio of OD260/OD280 ranged 1.9 to 2.1 could be used for successive analysis. The RNA samples were stored at –80 ◦C until further use. Total RNA was reversely transcribed as follows: 2.0 μg of the RNA isolated from each sample was added to a 25 μL reaction system containing 2.0 μL of oligodT18, 5.0 μL of dNTPs, 1.0 μL of RNase inhibitor, 1.0 μL of Moloney Murine Leukemia Virusreverse transcriptase (MMLV-RT), 5.0 μL of MMLV-RT reaction buffer (Promega) and RNase-free water. The parameters for the reverse-transcription procedure, based on the manufacturer's instructions (Promega), were as follows: 70 oC for 5 min followed by 42 ◦C for 2 h. The reverse transcribed products (cDNA) were stored at – 20 ◦C for PCR.

Real-time PCR of Relevant Genes

Gene expressions of the heat-stress protein (HSP) 70, GSH-Px, ornithine decarboxylase (ODC), epidermal growth factor (EGF) and epidermal growth factor receptor (EGFR) and β-actin was quantitatively detected using a relative quantitative real-time PCR technique. Quantitative real-time PCR analysis was carried out using the DNA Engine Mx3000PR fluorescence detection system (Stratagene) according to optimized PCR protocols and using a Brilliant SYBR Green QPCR Master Mix (Stratagene), containing a double-stranded DNAspecific fluorescent dye. All samples were measured in triplicate and β-actin were always amplified in parallel with the representative genes. The cDNA was subjected to real-time PCR using the primer pairs listed in Table 2. The real-time PCR reaction system (20 µL) contained 10 µL of SYBR Green qPCR mix, 0.3 µL of reference dye, 1 µL of each primer (both 10 µmol/L), and 1 µL of cDNA template (<10µg/L). Cycling conditions were 94 ◦C for 5 min, followed by 40 cycles of 94 ◦C for 30 s, 56 ◦C for 30 s and 72 ◦C for 40 s. Dissociation began with a step of 95 ◦C for 1 min, and then the melting curve from 55 to 95 ◦C at 0.2 ◦C/s was monitored continuously by fluorescence measurement. Expression levels were determined using the relative threshold cycle (CT) method as described by the manufacturer of the detection system. This was calculated for each gene by evaluating expression $2^{-\Delta\Delta CT}$, where ΔΔCT was the result of subtracting [CTgene – CTβ-actin](Heat-stressed) from [CTgene – CTβ-actin](Control). The error bars given represented the standard deviation of the CT values.

Metabolomic Analysis

Sample Preparation and Derivation

The analytes were derived and subsequently analyzed by gas chromatography – time of flight mass spectrometry (GC-TOFMS) following previously published protocols (Cheng et al. 2013). The serum samples were centrifuged at 3,000 × g for 15 min, and the supernatant collected was dried with N2 gas at ambient temperature. For jejunum mucosa sample preparation, adequate Zn/Si magnetic beads were added into the sample tube at first. Then, both for serum samples and jejunum mucosa samples, 500 mL of MeOH, 15 mL of ribitol solution (0.2 mg/mL in deionized water) as an internal standard, 15 mL of deionized water were added into the EP tube respectively and vortexed strongly. The samples were shaken at 100 × g in 70 ◦C for 15 min. The supernatant was taken out, into which amounts of 450 mL deionized water and 270 mL chloroform were added. The mixture was vortexed and then maintained at 80 × g and 37 ◦C for 5 min. The chloroform phase was discarded, and the water phase was collected and dried with N2 gas at 40 ◦C after centrifuged at 4,000 × g for 10 min. Methoxamine hydrochloride (40 mL, 20 mg/mL pyridine) was added to dissolve the residues and the resulting solution was incubated at 130 × g and 30 ◦C for 90 min to make oximation reactions complete. Then, BSTFA (with 1% TMCS, 40 mL) was added into the mixture and derivatization was performed at 37 ◦C for 30 min. Finally, the derived samples were maintained at ambient temperature for 120 min and stored at 4 ◦C for the next step.

GC-TOF-MS Analysis and Metabolites Determination

The derivatives were injected into DB-5 MS capillary column (30 m × 0.25 mm ID × 0.25 mm thickness) for separation in an Agilent 6890 N gas chromatographic system coupled with a Waters Micromass GCT mass spectrometer. The temperature of GC oven was first held at 70 ◦C for 0.1 min and then ramped at a rate of 5 ◦C per min to a final temperature of 310 ◦C with one minute hold time. Data was acquired under the following conditions: ionization mode, electron impact (EI); solvent delay, 5 min; electron impact energy, 70 eV; scan time, 0.87 s per scan and emission current, 60 mA. The temperature of injection, interface and ion source was set to 230, 290, and 220 ◦C, respectively. The scanning range of ion mass ranged from 50 m/z to 800 m/z. As a carrier gas helium was set at a constant flow rate of 1 mL/min.

Data Processing and Pattern Recognition

All GC-TOF-MS total ion current (TIC) chromatograms were analyzed by the MassLynx V4.1 (Waters). The acquired data were transformed and saved as SimcaP (x.txt) format file using the built-in MarkerLynx software package. The metabolites were identified by searching the National Institute of Standards and Technology database (NIST 2002 mass spectral database) with the corresponding mass spectrum and the available reference standards for all the detected peaks in the TIC chromatograms. Ribitol was used as the internal standard for normalization of the data to correct the variations during sample preparation and analysis. Multivariate pattern recognition analyses, such as PCA and PLS-DA, were employed to process the highdimension data from metabolomic study using Simca-P 11.5 software (Umetrics, Umea, Sweden) and MetaboAnalyst 2.0. Score plots were used to visualize the separation trend among the groups, and the VIP values of the variables in the loading scatter plot were used to reflect the variable importance and find out the potential biomarkers. The dataset was expressed as mean ± SE. The twotailed Student's t-test was used for statistical confirmation. $P < 0.05$ was considered significantly different. The metabolic functions of potential markers were interpreted using available biochemical database (KEGG, 2015).

RESULTS

Effects of PICB on the HSP70, GSH-Px, EGF, EGFR, ODC mRNA Expression of Jejunum Mucosa

The mRNA expressions of HSP70 on d 3 and d 7 after heat stress are shown in Figure 1. On d 3 after heat stress, the mRNA expression of HSP70 in groups 2–5 were significantly higher than group 1 ($P < 0.05$), among which group 4 had significantly higher mRNA expression than other groups ($P < 0.05$). On d 7 after heat stress, the mRNA expression of HSP70 showed similar trend as that on d 3, which demonstrated that the heat-stress model was successful and PICB could mitigate the effects of heat stress on chicks. The mRNA expressions of GSH-Px, EGF, EGFR and ODC on d 3 and d 7 after heat stress were shown in Figure 2. The mRNA expressions of GSH-Px in group 2–5 were greater than group 1 ($P < 0.05$) on d 3 after heat stress. On d 7 after heat stress, group 4 had the highest GSH-Px mRNA expression than other groups ($P < 0.05$). The mRNA expressions of EGF in group 4 and group 5 were significantly higher than group 1–3 ($P < 0.05$) both on d 3 and d 7 after heat stress. For the mRNA expression of EGFR, group 4–5 had higher values than group 2 and group 3 ($P < 0.05$), which in turn had higher values than group 1 ($P = 0.035$ and 0.046 respectively). There were similar trends for the values on d 3 and d 7 after heat stress. Group 3–5 had greater ODC mRNA expressions than group 1–2 ($P < 0.05$) on d 3 after heat stress, while on the seventh day, group 2–5 had similar mRNA expressions and all groups had higher mRNA expression than group 1. For all the parameters concerned, the mRNA expressions on d 7 after heat stress showed less alterations among groups than those on d 3 after heat stress and the group 4 showed higher values than other groups, which implied that PICB could improve the anti-oxidative and growth related parameters of heat-stressed chicks and the effects of heat stress and PICB would reduce as time went by. The samples of group 1 (control group) and group 4 on d 3 after heat stress were used for the subsequent metabolomic profiling analysis.

Metabolomic Alterations Induced by PICB Treatment on Heat Stressed Chicks

GC-TOF-MS TIC Chromatograms of Serum and Jejunum Mucosa Samples

The representative chromatograms were analyzed by GC-TOF-MS for serum and jejunum mucosa samples of heat-stressed broilers with and without PICB supplementation (Figure 3). Figure 3a and 3b represented TIC chromatograms of serum of heat-stressed chicks with and without PICB supplementation respectively, while Figure 3c and 3d represented those of jejunum mucosa. About 160 peaks were detected in the serum

and 240 peaks in the jejunum mucosa. Identification of individual peaks was performed via comparison of the corresponding mass spectrum in the National Institute of Standards and Technology (NIST) database using standard compounds for endogenous metabolites. A total of 40 compounds were identified in the serum and 44 in the jejunum mucosa. Most of these compounds were fatty and amino acids involved in lipid peroxidation, carbohydrate metabolism, and amino acid metabolism. It suggested that the basal metabolism of heat-stressed chicks could be different from those with PICB supplementation.

Multivariate Statistical Analysis of Metabolomic Data

All data containing the retention time, exact mass, and peak intensity were recorded for PCA and successively PLS-DA multiple statistical analyses. The score plots of PCA analyses for the metabolomic profiling were shown in Figure 4. The metabolomic profiles of heat-stressed chicks with PICB supplementation could be separated from those of heat-stressed chicks without PICB supplementation. The metabolomic profiles of jejunum mucosa could be separated clearly, while the serum metabolomic profiles couldn't be separated completely between the heat-stressed chicks with and without PICB supplementation. To verify the differences of metabolomic profiles of the 2 groups, the score plots analyzed by PLS-DA were shown in Figure 5. Using supervised multivariable statistical method, the weak separations between the two groups detected by PCA were sharpened and the Figure 5 demonstrated clear separation between the heat-stressed chicks with and without PICB supplementation both for serum and for jejunum mucosa metabolomic profiles. The PLS-DA loading scatter plots shown in Figure 6 were used to identify the potential different metabolites of heat-stressed chicks with and without PICB supplementation. The variable importance in the projection (VIP) values of the variables reflected their importance, and the variables with highest VIP values could be the potential biomarkers.

Differential Metabolites between Heat-Stressed Chicks with and without PICB Supplementation

Comparing the heat-stressed chicks with and without PICB supplementation, the first 10 most differential metabolites were identified for both serum and jejunum mucosa (Tables 3 and 4). The two-tailed Student's t-test showed serum glutamate in heat-stressed group was significantly higher than that in PICB group ($P < 0.05$). Serum serine in heat-stressed group tended to be higher than that in PICB group. Serum sorbitol in heat-stressed group tended to be lower than that in PICB group ($0.05 < P < 0.1$). For jejunum mocosa metabolomic profile, inositol and threitol in heat-stressed group were significantly higher than those in PICB group ($P < 0.05$), while serine, palmitic acid, L-ornithine were significantly lower than those in PICB group ($P < 0.05$).

DISCUSSION

mRNA Expressions of Anti-oxidative and Growth Promoting Related Genes

The gene expression profilings in brain, liver, leg muscles of heat-stressed broiler chicks were analyzed using gene microarrays and 6 genes were identified to be expressed differentially in the 3 tissues, including heat stress protein genes, apoptosis-related genes, cell proliferation and differentiation-related gene and the hunger and energy-metabolism related gene (Luo et al., 2014). Our previous study showed PICB could improve growth performance and antioxidant activity in serum of heatstressed broiler chicks (Dong et al., 2015), while Habibi et al. (2014) reported similar results using ginger products, which is another source of antioxidant compounds.

The gastrointestinal tract has been cited for its role in the heat stress of livestock and the normal morphology and integrity of the small intestine are important for optimum growth and production in poultry (Sun et al., 2015). In this paper, PICB was found to have positive influences on the mRNA expressions of anti-oxidative and growth-related genes in jejunum mucosa. HSP70 had various biological functions such as cell protection, antiapoptosis and anti-oxidation (Kiang and Tsokos, 1998). The heat

resistance capacity of cells or organs was positively correlated with the HSP70 level (Binder et al., 2001 and Kilgore et al., 1994), which is highly dependent with the mRNA expression regulation (Nagata et al., 1998). In this paper both PICB and VC had higher HSP70 mRNA expression levels than the control group and group 4 had highest value among the groups, which showed high protection capacity of PICB against heat stress.

Heat stress can cause the body produce excess reactive oxygen species (ROS), which could significantly affect animal antioxidant status and lipid peroxidation levels (Gu et al., 2008). The enzymes with antioxidative activities are especially important for heatstressed animals. GSH-Px is one of main enzymes that scavenge ROS using GSH as substrates to eliminate the excess H2O2 produced in the body. The mRNA expressions of GSH-Px of both VC and group 4 was found up-regulated significantly in this study, which was in accordance with the higher GSH-Px activity in the serum and liver (Liu et al., 2011; Dong et al., 2015). VC is a common antioxidant used in feed industry. These results implied that PICB has higher antioxidative capacity than VC. Regarding to the growth promoting genes, mRNA expressions of EGF, EGFR and ODC were studied. EGF and EGFR had multi-functions by stimulating the cell mitosis and differentiation (Dvorak et al., 1998), regulating cell growth and proliferation, repairing the injured tissue, and playing important role in mucosa and gland recovery and mucosa integrity (Tamawski et al., 1995). In this study, mRNA expressions of EGF and EGFR were improved by PICB in heated stressed broilers, and similar results were obtained in the research of recovery mechanism of the heat-stressed swine intestinal mucosa (Liu et al., 2009). VC improved the mRNA expressions of EGFR, while had little effect on EGF mRNA expression comparing with the control group, which show VC could have some growthpromoting effects with limited capacity comparing with PICB. ODC was the first limiting enzyme in the synthesis of polyamines, which was essential in the cell growth, proliferation, differentiation and ROS scavenges (Says et al., 2006). ODC played an important role in the recovery of injured intestinal mucosa. Some Traditional Chinese Medicine can improve the mRNA expression of ODC in injured intestinal mucosa (Zhao, 2012; Kong et al., 2009), so as to increase the ODC activity and polyamine content. In this study, mRNA expression of ODC was up-regulated in heat-stressed broiler chicks with PICB or VC supplementation comparing with those without PICB, which could lead to the better growth performance and oxidative status of birds.

Dong et al. (2015) reported PICB could improve T-SOD and GSH-Px activities and MDA content of chicks blood. It also improved average daily gain and average feed daily intake while decreased feed conversion rate of heat-stressed chicks. This study resulted that the mRNA expression alterations of GSH-Px, ODC, EGF and EGFR revealed the effects of PICB on heats stressed broilers at gene level, which could be the intrinsic reason of birds' growth and oxidative status performance reported in our previous study.

Systematic Analysis of the Effects of PICB on Heat Stressed Broiler Chicks

Gene expression and enzymes activity studies showed that heat stress influenced greatly the growth and oxidative related factors and PICB supplementation could restore the alterations. To bridge the gap between gene expression and physiological responses, metabolomic profilings of serum and jejunum mucosa were studied on the heat-stressed broilers with or without PICB supplementation. Three biomarkers in serum and 5 in jejunum mucosa were identified which could reveal the multi-pathway metabolic perturbation associated with heat stress and PICB treatment in broilers. After researching on the KEGG database (KEGG, 2015), the 7 biomarkers (serine was identified both in serum and in jejunum mucosa) were distributed among the metabolism pathways of the glutathione metabolism (Glutamate, L-ornithine), amino acid metabolism (serine, L-ornithine), lipid metabolism (palmitic acid), carbohydrate metabolism (sorbitol), GnRH signaling pathway (inositol), etc., while there were no metabolic pathway information about threitol in KEGG website.

Perturbations of Redox Status

Glutamate has an important derivative, glutamine, which has strong antioxidative capacity. Glutamine exerts its ability of anti-oxidant mainly in 2 ways: 1) oxidation as an energy material or 2) as a precursor of glutathione reducing oxidative stress. The oxidation of glutamine eliminated strong oxidizing substance in cell and protect other important cell components from oxidative damage (Wang et al., 2004). From another point of view, glutamate is an important and excitable amino acid neurotransmitter in central nervous system. Under normal physiological state, the synthesis, decomposition, intake and absorption of glutamate is a process of dynamic equilibrium. When the balance is destroyed, the excitable glutamate toxicity that may cause various diseases in central nervous system. The content of glutamate increased significantly under acute heat stress (Reznikov et al., 2007).

In this study, the glutamate content in the serum was high under heat stress, which could be the main reason that most boilers demonstrated typical heat stress syndrome such as depression, stretching neck with open mouth, fever panting, accelerated breathing etc. PICB could accelerate the transformation from glutamate to glutamine to exert its anti-oxidative function so as to reduce glutamate content in the serum remarkably, which would attenuate the heat stress syndrome and improve the redox status of heat-stressed broilers. L-ornithine also influenced the glutathione metabolism. In this study, the increased level of L-ornithine in PICB treated chicks jejunum mucosa could stimulate the glutathione metabolism to increase the anti-oxidative capacity of heated stressed chicks (Wang et al., 2008).

Perturbations of Growth Performance

PICB supplementation altered several growth related metabolites in heat-stressed chicks. Serine is an unnecessary amino acid and participated glycine, serine and threonine metabolism, cysteine and methionine metabolism, mucin type O-glycan biosynthesis, adipocytokine signaling pathway, insulin signaling pathway, glycosaminoglycan biosynthesis - heparan sulfate/heparin. It works on the lipid/fatty acid metabolism, muscle growth and immune system. In the metabolomic study, serine was found to have increased level in jejunum mucosa while decreased level in serum in PICB treated chicks comparing the control group, which revealed PICB promote the serine synthesis in jejunum mucosa and utilization in the serum, so as to improve the growth performance. Sorbitol mainly influenced energy metabolism and works on galactose metabolism, fructose and mannose metabolism, phosphotransferase system (PTS), and ABC transporters. The higher content in serum reflect the higher energy metabolism in PICB treated chicks comparing to the control group. Inositol had similar function as Vitamin B and biotin and was necessary substances for growth of human and microorganisms. KEGG showed inositol involved in GnRH signaling pathway, biosynthesis of secondary metabolites, regulation of actin cytoskeleton, and glycerophospholipid metabolism. Inositol had physiological functions in the synthesis of proteins and lipid metabolism, cell membrane formation and cell messenger regulating calcium balance and transfer (Zhang et al., 2007). In this study, the inositol content in jejunum mucosa of PICB group is lower than the control group. It could be explained that the broilers under heat stress had less capacity to absorb inositol while PICB could improve the absorption of inositol, which would exert its physiological functions. Palmitic acid was involved in biosynthesis of unsaturated fatty acids, fatty acid metabolism, and cutin, suberine, and wax biosynthesis, and it can promote endothelial progenitor cells' apoptosis through p38 and JNKMARKs pathway (Jiang et al., 2010). It also influenced the function of proteins by protein palmitoylation (Mitehell et al., 2006). The results of this study suggested PICB prevent the absorption of palmitic acid, which could mitigate the apoptosis of broiler organs caused by heat stress.

L-Ornithine was one of the important intermediate metabolites in urea cycle and the metabolism of proline, glutamate, arginine and polyamine. L-Ornithine could stimulate pituitary to secretion growth hormone, and promote protein synthesis and catabolism of sugar and fat (Wang et al., 2008). The plasma concentration of uric acid increased in heat-stressed broilers comparing to the control group and more L-ornithine was needed to get rid of the excess uric acid (Sun et al., 2015). In this study chicks with PICB supplementation produced more ornithine after feed intake and digestion, consequently, could get rid of

uric acid and improve the growth performance. However, the effects of PICB supplementation to the heat-stressed chicks were far beyond the effects on anti-oxidative parameters and growth performance and further studied were needed to understand the functions of PICB on heat-stressed broilers.

In conclusion, significantly changes of HSP70 mRNA expression of heat-stressed chicks showed that the heat stress model established in this study is successful. The addition of PICB in diet can improve significantly mRNA expressions of some anti-oxidative and growth parameters. The metabolomic profiling study confirmed PICB could alter the basal metabolism of heat-stressed chicks showing its beneficial effects of anti-oxidative property and growth promoting effect. The novel approach combining the mRNA expressions with metabolomic study can provide the integrative and systematic insight of the mechanism of PICB function.

ACKNOWLEDGMENTS

This work was partially supported by the Science and Technology Projects of Beijing Municipal Commission of Rural Affairs (20150125), the Project of Construction of Innovative Teams and Teacher Career Development for Universities and Colleges under the Beijing Municipality (PXM2013014207000067). The Importation and Development of High-Caliber Talents Project of Beijing Municipal Institutions (CIT&TCD201304093) and the State Key Laboratory of Animal Nutrition of Beijing Institute of Animal Husbandry and Veterinary Medicine, Chinese Academy Agricultural Science (2004DA125184F130). The authors thank the State Key Laboratory of Animal Nutrition of Beijing Institute of Animal Husbandry and Veterinary Medicine, Chinese Academy Agricultural Science for providing experiment base.

TABLES & FIGURES

Table 1. Composition and nutrient levels of basic diets, %.

Ingredients	Content	
	1 to 3 weeks	4 to 6 weeks
Corn	59.4	61.9
Soybean meal (46%CP)	25	22.4
Fish meal (68% CP)	8	7
Soybean oil	4	5.5
Limestone	1.2	1.3
$CaHPO_4$	1	0.5
NaCl	0.3	0.3
Premix[1]	1	1
DL-Met	0.1	0.1
Total	100.00	100.00
Nutrient levels[2]		
ME(MJ/kg)	3.12	3.19
CP	22.05	20.23
Ca	1.02	0.93
AP	0.48	0.39
Lys	1.25	1.13
Met	0.49	0.46

[1]Premix provided the following per kilogram of diets: Zn (as zinc sulfate) 75 mg, Fe (as ferrous sulfate) 100 mg, Mn (as manganese sulfate) 100 mg, Cu (as copper sulfate) 8 mg, I (as potassium iodide) 0.45 mg, Se (as sodium selenite) 0.3 mg, vitamin A 8800IU, vitamin D3 3,000 IU, vitamin E 30 IU, vitamin K 1.65 mg, vitamin B12 22 μg, vitamin B1 1.1 mg, vitamin B 26.6 mg, pantothenate 11 mg, vitamin B3 nicotinic 66 mg, vitamin B6 4.4 mg, biotin 0.15 mg, choline 1,300 mg.

[2]Values calculated based on NRC (1994) ingredient composition and NY/T33–2004 AA broilers nutrient requirements.

Table 2. Primers used for real-time PCR.

Gene name[1]	Primer set	Serial Number	Expected size (bp)
HSP70	F: 5′- ATGCTAATGGTATCCTGAACG-3′ R: 5′- TCCTCTGCTTTGTATTTCTCTG-3′	NM_005345.5	145
ODC	F: 5′- GCCCGAGCTTACACTCAAGTC-3′ R: 5′- GGCAGTAAAGCCCTCGTCAA-3′	NM_001167766.1	111
GSH-Px	F: 5′- AAGAACTTCGGGCACCAGGA-3′ R: 5′- TGTACTGCGGGTTGGTCATC-3′	NM_001277853.1	210
EGF	F: 5′- CAATGCTCGGTTTGCGGTTG-3′ R: 5′- GGTTCGCTGTCTCACACTCA-3′	NM_001001292.1	86
EGFR	F: 5′- GGTTGGTCTAGGCATCGGTCT-3′ R: 5′- TGGTTCGACAAGCTCCCTCT-3′	NM_00205497.2	97
β-actin	F: 5′- CCGTGCTGTGTTCCCATCT-3′ R: 5′- TGCTCTGGGCTTCATCACC-3′	NM_001613.2	101

[1]HSP70: Heat Stress Protein 70; GSH-Px: Glutathione Peroxidase; ODC: Ornithine Decarboxylase; EGF: Epidermal Growth Factor; EGFR: Epidermal Growth Factor Receptor.

Table 3. The first ten differential metabolites in serum of heat-stressed chicks with or without PICB supplementation.

Order	Retention time	Metabolites	Control group	PICB group	*P*-value	Fold change
1	18.706	Glutamate	4455.19 ± 396.37	2044.02 ± 699.96	0.04	0.46
2	14.023	β-alanine	1116.87 ± 352.76	684.98 ± 138.21	0.32	0.61
3	12.389	Serine	8319.08 ± 413.10	6424.13 ± 468.11	0.07	0.77
4	16.322	5-oxoproline	10778.15 ± 1051.17	7684.72 ± 1844.55	0.22	0.71
5	10.739	Proline	3337.21 ± 270.66	2836.77 ± 782.55	0.58	0.85
6	37.257	Disaccharide	5085.89 ± 4144.78	3923.36 ± 879.42	0.80	0.77
7	26.273	Glucopyranose	10465.17 ± 1088.03	13024.44 ± 494.46	0.10	1.24
8	13.006	Threonine	5783.96 ± 123.71	7155.18 ± 855.26	0.19	1.24
9	23.640	Spermine	8296.91 ± 1284.94	9345.66 ± 963.98	0.55	1.13
10	25.123	Sorbitol	5322.81 ± 1459.74	9536.88 ± 594.58	0.06	1.79

PICB: Polyphenolic extract of the involucres of Castanea mollissima Blume.

Table 4. The first 10 differential metabolites in jejunum mucosa of heat-stressed chicks with or without PICB supplementation.

Order	Retention time	Metabolites	Control group	PICB group	*P*-value	Fold change
1	23.086	Inositol	33504.56 ± 2518.78	16216.89 ± 5219.32	0.04	0.48
2	17.701	Xylulose	2904.81 ± 226.01	2028.80 ± 208.23	0.11	0.70
3	26.419	Galactonic acid	11100.61 ± 2340.60	3727.71 ± 1852.78	0.15	0.34
4	16.035	Threitol	3335.80 ± 129.11	978.49 ± 418.04	0.01	0.29
5	12.384	Serine	4459.64 ± 490.41	7052.43 ± 699.89	0.05	1.58
6	10.684	Isoleucine	9142.94 ± 1389.53	9850.29 ± 1477.93	0.75	1.08
7	10.151	Leucine	5913.88 ± 183.82	6648.32 ± 394.97	0.17	1.12
8	27.552	Palmitic acid	5192.43 ± 147.77	9012.98 ± 257.79	0.01	1.74
9	23.635	L-Ornithine	2259.78 ± 663.82	4843.36 ± 449.31	0.04	2.14
10	8.734	Valine	5554.58 ± 735.19	6600.18 ± 576.79	0.33	1.19

PICB: Polyphenolic extract of the involucres of *Castanea mollissima* Blume.

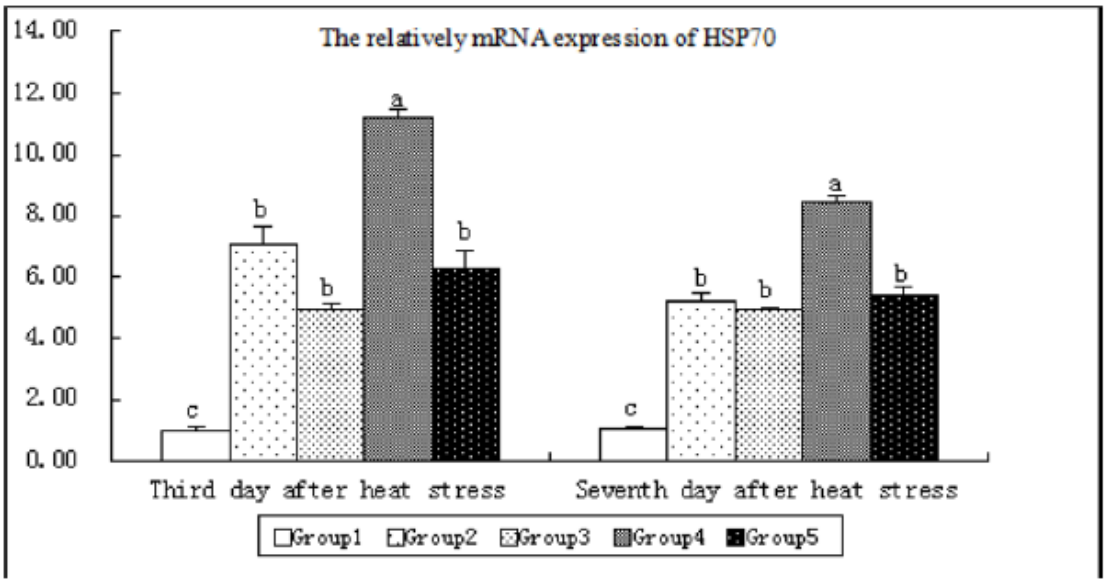


Figure 1. The mRNA expressions of HSP70 on d 3 and d 7 after heat stress. HSP70: heat stress protein 70; Group 1: chicks fed with the basal diet; Group 2: chicks fed with the basal diet supplemented with VC; Groups 3–5: chicks fed with the basal diet supplemented with 0.2%, 0.3% and 0.4% Polyphenolic extract of the involucres of *Castanea mollissima* Blume respectively; Value columns with different lowercase letter mean significant difference ($P < 0.05$).

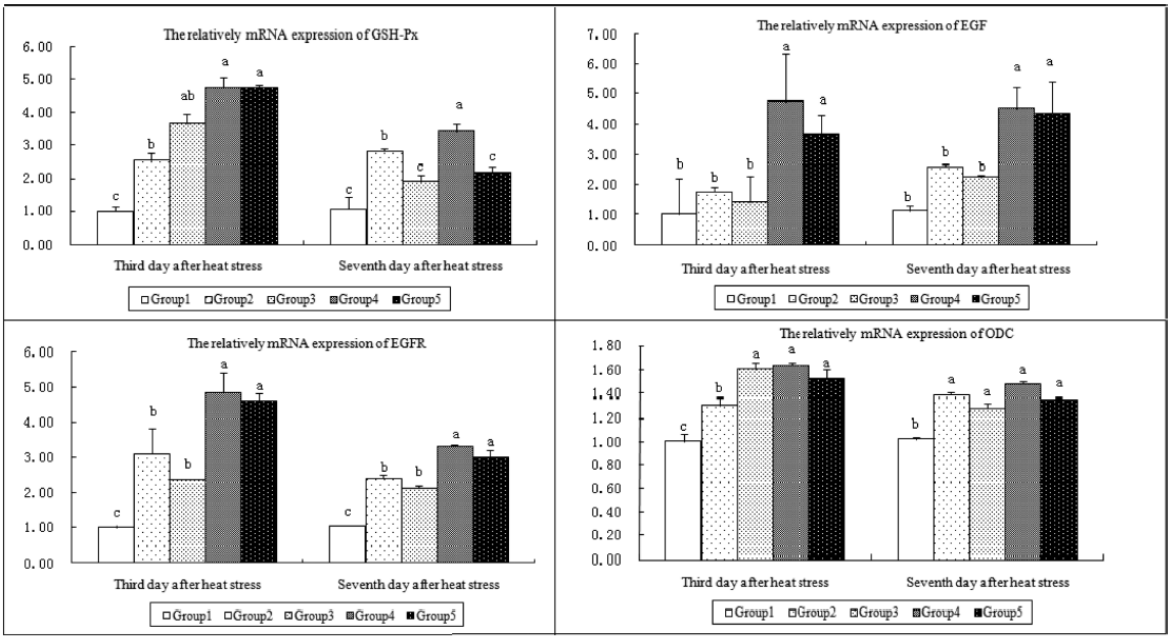


Figure 2. The mRNA expressions of GSH-Px, EGF, EGFR and ODC on d 3 and d 7 after heat stress. GSH-Px: Glutathione Peroxidase, EGF: Epidermal Growth Factor, EGFR: Epidermal Growth Factor Receptor, ODC: Ornithine Decarboxylase; Group 1: chicks fed with the basal diet, Group 2: chicks fed with the basal diet supplemented with VC, Group 3–5: chicks fed with the basal diet supplemented with 0.2%, 0.3%, and 0.4% polyphenolic extract of the involucres of *Castanea mollissima* Blume respectively; Value columns with different lowercase letter mean significant difference ($P < 0.05$).

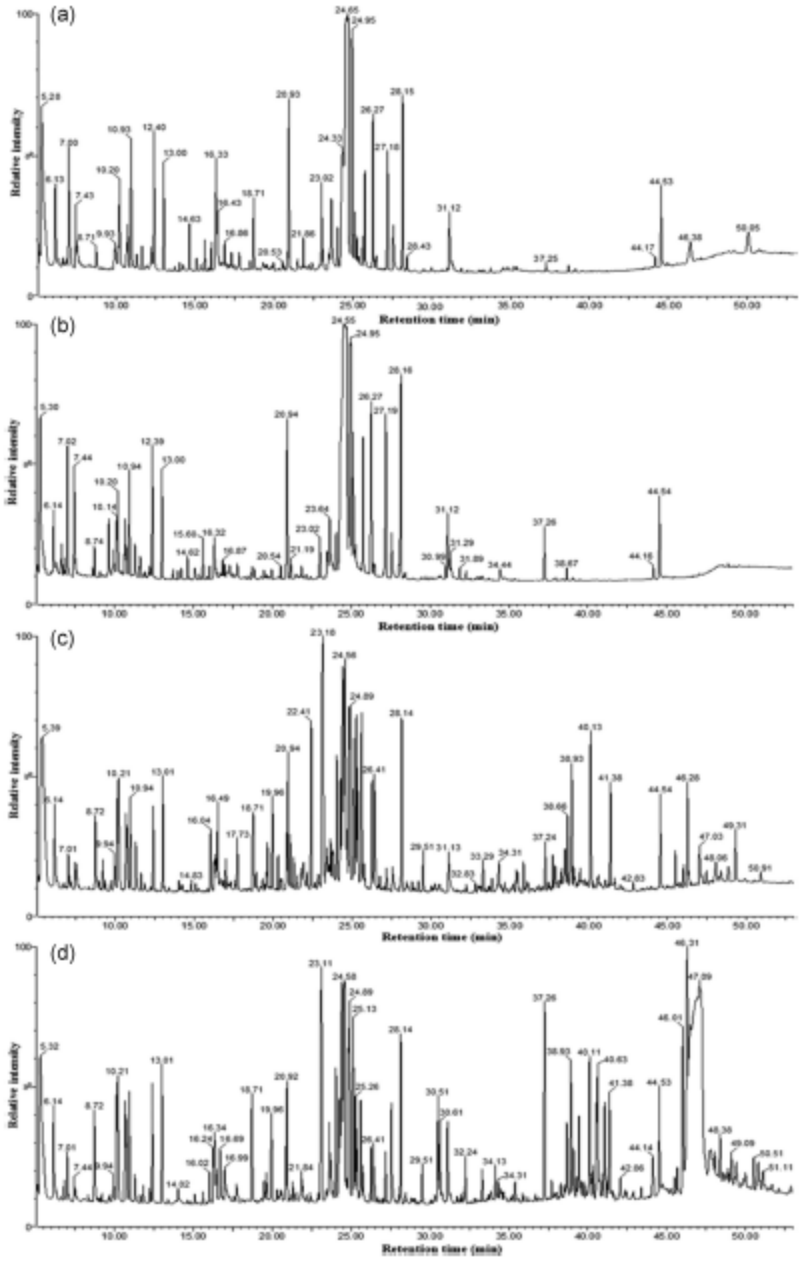


Figure 3. Comparison of GC-TOF-MS total ion current (TIC) chromatographs of heat-stressed chicks treated with PICB. PICB: Polyphenolic extract of the involucres of *Castanea mollissima* Blume; a: serum of control group, b: serum of PICB group, c: jejunum mucosa of control group, d: jejunum mucosa of PICB group. In a and b, the peaks (corresponding retention time, min) represent, in sequence, Lactic acid (5.305), Alanine (6.138), Oxalic acid (7.005), Hydroxybutyric acid (7.438), Phosphoric acid (10.205), Isoleucine (10.655), Proline (10.739), Glycine (10.939), Succinic acid (11.306), Glycerol acid (11.622), Serine (12.389), Threonine (13.006), 2-hydroxybutyrate (13.706), β-alanine (14.023), Aspartic acid (15.106), Malic acid (15.606), Threitol (16.039), 5-oxoprolin (16.322), Hydroxyproline (16.422), Erythronic acid (16.872), α-amino acid (17.789), Glutamate (18.706), Adonitol (internal standard)(20.939), Ornithine (21.486), Single glycerol phosphate (21.856), Isocitric acid (23.023), Spermine (23.64), Fructose (24.04). The unknown sugar 1 (24.323), Glucose (24.956), Sorbitol (25.123), Gluconic acid (25.273), Turanose (25.756), Glucopyranose (26.273), Unknown peak (27.19), Palmitic acid (27.556), Inositol (28.157), Stearic acid (31.124), Disaccharide (37.257), Cholesterol (44.158). In c and d, the peaks (corresponding retention time, min) represent in sequence Lactic acid (5.318), alanine (6.151), Oxalic acid (7.017), Hydroxybutyric acid (7.451), Valine (8.734), Leucine (10.151), Glycerol (10.218), Isoleucine (10.684), Proline (10.751), Glycine (10.951), Succinic acid (11.301), Glycerol acid (11.634), Serine (12.384), Threonine (13.018), β-alanine (14.018), Aspartic acid (15.085), Threitol (16.035), Methionine (16.252), 5-oxoproline (16.335), Hydroxyproline (16.435), Creatinine (17.002), Xylulose (17.701), Glutamate (18.701), Ribose (19.952), Xylitol (20.302), Adonitol (internal standard)(20.935), Putrescine (21.068), Glucose acid (22.152), Fructose (22.802), The unknown sugar 1 (23.045), Inositol (23.086), Ornithine (23.635), Galactose (23.769), Glucose (24.552), Glucopyranose (26.269), Galactitol (25.152), Sorbitol (25.285), Galactonic acid (26.419), Gluconic acid (26.752), Palmitic acid (27.552), 9,12-octadecadienoic acid (30.503), Stearic acid (31.119), Glycerol galactose (31.702), Disaccharide (32.253).

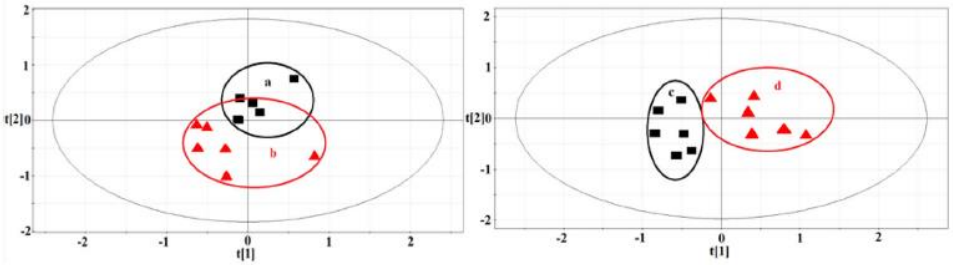


Figure 4. PCA score plot of the GC-TOF-MS data collected from heat-stressed chicks with or without PICB supplementation. PICB: Polyphenolic extract of the involucres of *Castanea mollissima* Blume; a: serum of heat-stressed group, b: serum of PICB group, c: jejunum mucosa of heat-stressed group, d: jejunum mucosa of heat-stressed group.

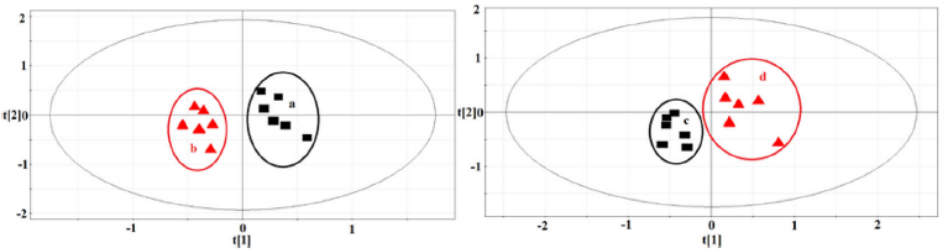
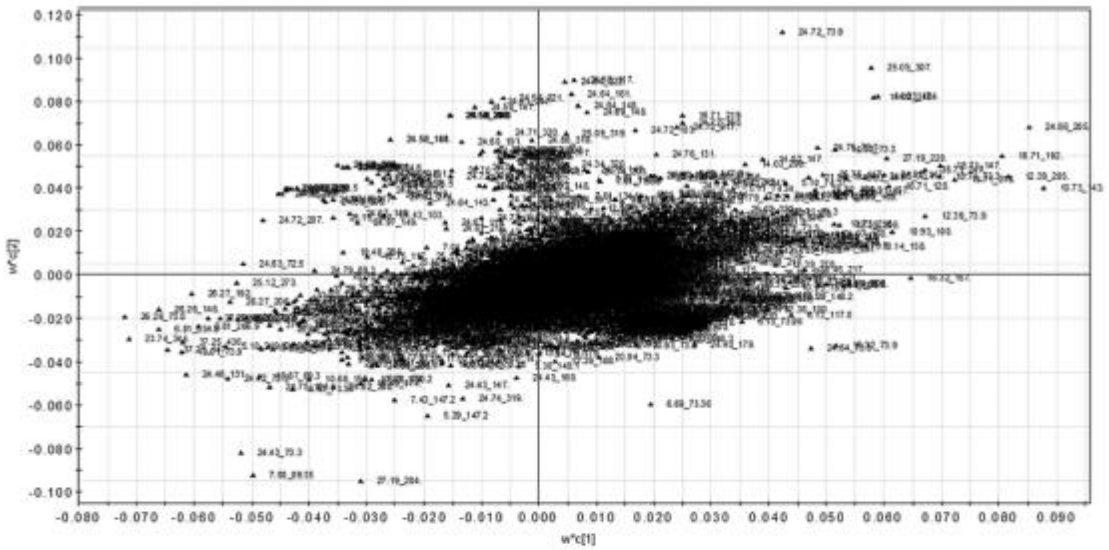
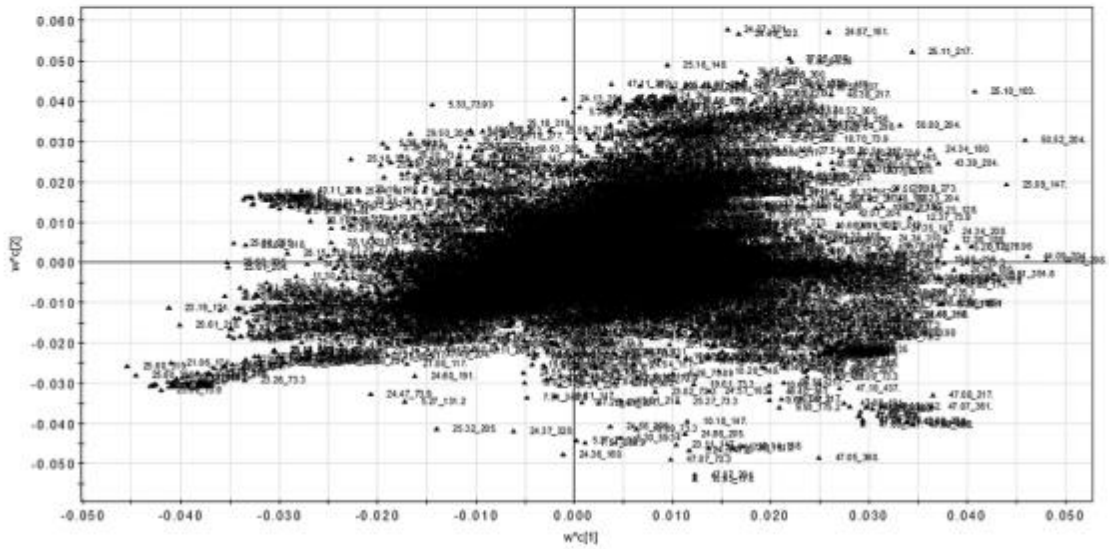


Figure 5. PLS-DA score plot of the GC-TOF-MS data collected from heat-stressed group with or without PICB supplementation. PCA: Principal Component Analysis, PGC-TOF-MS: gas chromatography – time of flight - mass spectrometry. PICB: Polyphenolic extract of the involucres of *Castanea mollissima* Blume; a: serum of heat-stressed group, b: serum of PICB group, c: jejunum mucosa of heat-stressed group, d: jejunum mucosa of heat-stressed group.



PLS-DA loading plots of serum



PLS-DA loading plots of jejunummucosa

Figure 6. PLS-DA loading scatter plot of the GC-TOF-MS data collected from heat-stressed chicks with or without PICB supplementation.

REFERENCES

Binder, R. J., N. E., Blachere, and P. K. Srivastava. 2001. Heat shock protein chaperoned peptides but not free peptides introduced into the cytosol are presented efficiently by major histompatibility complex I molecules. J. Biol. Chem. 276:17163–17171.

Cheng, J. H., N. Y. Che, H. J. Li, K. P. Ma, S. M. Wu, J. J. Fang, R. Gao, J. X. Liu, X. Z. Yan, C. Y. Li, and F. T. Dong. 2013. Gas chromatography time-of-flight mass spectrometrybased metabolomic analysis of human macrophages infected by M. tuberculosis. Anal. Lett. 46:1922–1936.

Dong, S., H. Li, K. J. Guo, L. Gasco, and I. Zoccarato. 2015. Anti-oxidative activity of the polyphenols from the involucres of Castanea mollissima Blume and their mitigation effects on heat stress. Poult. Sci. 945:1096–1104.

Dvorak, B., J. Kolinsk, D. L. McWilliam, C. S. Williams, T. Higdon, M. Z´akosteleck´a, and O. Koldovsk´y. 1998. The expression of epidermal growth factor and transforming growth factor-A mRNA in the small intestine of suckling rats: organ culture study. FEBS Lett. 435:119–124.

Gu, X. H., S. S. Li, and H. Lin. 2008. Effects of hot environment and dietary protein level on growth performance and meat quality of broiler chickens. Asian-Australas. J. Anim. Sci. 21:1616–1623.

Habibi, R., G. H. Sadeghi, and A. Karimi. 2014. Effect of different concentrations of ginger root powder and its essential oil on growth performance, serum metabolites and antioxidant status in broiler chicks under heat stress. Br. Poult. Sci. 55:228–237.

Insurk, J., K. YoungHyun, M. YangSoo, and S. SeaHwan. 2014. Effects of vitamin C or E on the pro-inflammatory cytokines. heat shock protein 70 and antioxidant status in broiler chicks under summer conditions. J. Anim. Sci. 27:749–756.

Jiang, H., C. Liang, and X. Liu. 2010. Palmitic acid Promotes endothelial progenitor cells apoptosis via p38 and JNK mitogen-activated Protein kinase pathways. Atherosclerosis. 210:71–77.

Jouany, J. P., and D. P. Morgavi. 2007. Use of ‘natural’ products as alternatives to antibiotic feed additives in ruminant production. Animal. 1:1443–1466. KEGG, 2015. http://www.kegg.jp/kegg. Accessed on April 15, 2015.

Kiang, J. G., and G. C. Tsokos. 1998. Heat shock protein 70 kDa: Molecular biology, biochemistry and physiology. Pharmacol. Ther. 80:183–201.

Kilgore, J. L., B. F. Tmson, and D. K. Saunders. 1994. Stress protein induction in skeletal muscle comparison of laboratory models to naturally occurring hypertrophy. J. Appl. Physiol. 76:598–601.

Kong, X. Y., B. W. Wen, and W. Chen. 2009. The influence of chemical component of Radix codonopsis and Astragalus polyaccharin for IEC-6 cells proliferation, ODC and hephaestin expression. Trad. Chinese Med. 32:407–410.

Li, H., S. Dong, M. C. Gu, and K. J. Guo. 2015. Effect of chestnut involucres polyphenols on growth performance and antioxidant properties of AA broilers. Sci. Agric. Sinica. 48:788–795.

Liu, F., J. Yin, M. Du, P. Yan, J. Xu, X. Zhu, and Ju . 2009. Heatstress-induced damage to porcine small intestinal epithelium associated with downregulation of epithelial growth factor signaling. J. Anim. Sci. 87:1941–1949.

Liu, H. W., X. F. Dong, J. M. Tong, and Q. Zhang. 2011. A comparative study of growth performance and antioxidant status of rabbits when fed with or without chestnut tannins under high ambient temperature. Anim. Feed Sci. Tech. 164: 89–95.

Liu, Y., Z. B. Lin, G. G. Tan, Z. Y. Chu, Z. Y. Lou, J. P. Zhang, Z. Y. Hong, and Y. F. Chai. 2013. Metabonomic studies on potential plasma biomarkers in rats exposed to ionizing radiation and the protective effects of Hong Shan capsule. Metabolomics. 9:1082– 1095.

Luo, Q. B., X. Y. Song, C. L. Ji, X. Q. Zhang, and D. X. Zhang. 2014. Exploring the molecular mechanism of acute heat stress exposure in broiler chickens using gene expression profiling. Gene. 546:200– 205.

Mitehell, D. A., A. Vasudevan, and M. E. Linder. 2006. Protein palmitoylation by a family of DHHC protein S-acyl transferases. J. Lipid Res. 47:1118–1127.

Nagata, K., M. Satoh, and A. D. Miller, 1998. Involvement of Hsp 47 in the folding and processing of procollagen in the endoplasmic reticulum. Molecular Chaperones in the Life Cycle of Proteins. New York: Dekker. 10:225–240.

Nicholson, J. K., and J. C. Lindon. 2008. Systems biology: metabonomics. Nature. 455:1054–1056.

Reznikov, L. R., C. A. Gri1lo, and G. G. Piroli. 2007. Acute stress mediated increases in extracellular glutamate levels in the rat amygdala: differential effects of antidepressant treatment. Eur. J. Neurosci. 25:3109–3114.

Says, I. G., V. Battaglia, and C. A. Rossi. 2006. Free radical scavenging action of the natural polyamine spermine in rat liver mitochondria. Free Radic. Biol. Med. 41:1272–1281.

Schiavone, A., K. J. Guo, S. Tassone, L. Gasco, E. Hernandez, R. Denti, and I. Zoccaratol. 2008. Effects of a natural extract of chestnut wood (SILVAFEED ENC) on digestibility, performance traits and nitrogen balance of broiler chicks. Poult. Sci. 87:521– 527.

Shi, E. H., K. J. Guo, H. Li, M. C. Gu, L. Lu, and C. X. Jia, 2013. Castanea mollissima Blume polyphenols: extraction technology optimization and its antioxidant activity. Chin. J. Anim. Nutr. 25:406–414.

Sun, X. L., H. C. Zhang, A. Sheikhahmadi, Y. F. Wang, H. C. Jiao, H. Lin, and Z. G. Song, 2015. Effects of heat stress on the gene expression of nutrient transporters in the jejunum of broiler chickens (Gallus gallus domesticus). Int. J. Biometeorol. 59:127–135.

Tamawski, A., K. Tanoue, and A. M. Santos. 1995. Cellular and molecular mechanisms treatment. Scand. J. Gastroenterol. Suppl. 210 (Suppl1):79.

Wang, L., X. L. Piao, S. W. Kim, X. S. Piao, Y. B. Shen, and H. S. Lee. 2008. Effects of Forsythia suspensa extract on growth performance, nutrient digestibility, and antioxidant activities in broiler chickens under high ambient temperature. Poult. Sci. 87:1287– 1294.

Wang, X. B., F. L. Li, and D. F. Li. 2004. The antioxidant effect of free glutamine in body. Biophys. J. 20:429–433.

Zhang, T. X., Z. W. Li, and B. T. Zhang. 2007. The nature of inositol and its application in aquaculture. Feed Industry. 14:28–30.

Zhao, S. Q., 2012. The research of Radix glycyrrhizeae and Radix codonopsis for stress ulcer and polyamine in rats. Guangzhou University of Chinese Medicine. 2012:1–79.