Metabolomics Approach Reveals Diagnostic Biomarkers and Metabolic Changes in Heat-Stressed Dairy Cows

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Abstract: This study was planned to investigate the effect of heat stress on body weight, physiological parameters, milk yield, metabolome of milk and plasma in dairy cows by using Hydrogen-1Nuclear Magnetic Resonance Spectrometry (1HNMR)-based metabolomics approach. Ten Holstein cows in heat stress period (28 d) and another ten Holstein cows in thermo-neutral period (28 d) were maintained at the same feeding and management regime. Cows under both treatments were similar in parity, body weight, and days in milk at the beginning of each period. Bodyweight of each cow was recorded at the beginning and end of each period. Milk production, rectal temperature, and respiration rate were recorded weekly under each treatment while Blood samples and milk samples for 1HNMR analyses were collected at the end of each period. Cows in heat stress period had greater respiration rates (p<0.01), greater rectal temperatures (p< 0.01), lower milk yield (p< 0.01) and lower milk protein percentage (p<0.05) than cows in thermo-neutral period. Cows in heat stress period exhibited the greater milk concentrations of N-acetyl glycoprotein (NAG), choline, scylloinositol, pyridoxamine and lower milk concentrations of O-acetyl glycoprotein (OAG), citrate, glycerophosphocholine (GPC) and methyl phosphate than those in thermo-neutral period. Besides, cows in heat stress period showed greater plasma concentrations of alanine, glutamate, glucose, urea, histidine, 1-methylhistidine (1-MH) and formate. However, the plasma concentration of very-low-density lipoprotein (VLDL), low-density lipoprotein (LDL), lipid, leucine, and 3-hydroxybutyrate (3-HB) were lower as compared to cows in the thermo-neutral period. In conclusion, heat stress affects the metabolites in milk and plasma in dairy cows. It is reflected by the alteration of 8 metabolites in milk and 12 metabolites in blood plasma. Which are mainly involved in gluconeogenesis, proteolysis, and milk fatty acid synthesis. Because of their capability to hit multiple targets, these metabolites could be as the potential biomarkers for dairy cows undergoing heat shock. The biological significances of the metabolite changes in the milk and plasma were also analyzed.

Keywords: metabolomics; dairy cow; biomarkers; nuclear magnetic resonance

1. Introduction

Heat stress in animals is caused by the imbalance between heat load and heat dissipation when
ambient temperature and humidity exceed certain limits and is one of the significant challenges faced by modern dairy cow farming. Genetic selection for high milk yield [1], together with the increased global temperature [2], further exacerbates the heat stress of dairy cow. The problems encountered by the heat-stressed dairy cow include impaired milk production and milk quality, metabolic disorders, rumen acidosis, negative energy balance, and even death [3-5]. Some blood metabolites of dairy cows were reported to be changed during heat stress [6-8]. However, no studies concerning the effect of high temperature on the plasma metabolome, and its relationship with milk production of mid-lactating dairy cows have been reported yet.

Metabolomics, a useful tool for analyzing the changes of metabolites in physiological fluids and tissues in response to internal and external stimulations, has been successfully applied for filtering out the biomarkers for milk quality [9], energy metabolism [10] and rumen health in dairy cows [11]. However, only a few studies attempting to identify biomarkers for diagnosis of heat stress of dairy cows based on milk [12] and plasma [13] metabolomics have been reported. Nuclear magnetic resonance (NMR)-based metabolomics is one of the frequently used techniques for metabolomics studies [14]. This method provides an overview of the ongoing metabolic processes in the organism and may illustrate the mechanism responsible for alterations in milk yield. The NMR-based metabolic profiling of serum, milk, cells, and tissue extracts have yielded valuable information by assessing drug safety, diagnosing ketosis in dairy cows, characterizing diet-induced and heat stress-induced variation [15, 16]. In this study, we formulate the hypothesis that dairy cows suffering from heat stress may manifest signs of blood metabolite changes which can be used as biomarkers for evaluating the influences of heat stress on dairy cows and for revealing the metabolic changes during heat stress. The current study is conducted to screen the potential biomarkers of heat stress exposure, to find the altered metabolic pathways and to identify whether the metabolic changes were related to milk yield reduction in heat-stressed dairy cow, by comparing the metabolome changes in milk and plasma between cows in heat stress and those under thermo-neutral condition, using NMR-based metabolomic approach.

2. Materials and Methods

The experimental protocol involved in the present study was approved by the Animal Care Advisory Committee of Sichuan Agricultural University (Animal Ethics Committee approval number is CD-SYXXK-2017-015).

2.1 Animal and Experimental Design

This study was carried out at Mei jiadun Dairy Cow Farm, Huang gang, Hubei province; China. Experiment consisted of two periods, one in spring (from mid-March to mid-April in 2014) as thermo-neutral period and other in summer (from mid-July to mid-August in 2014) as heat stress period. Each period consisted of a 10d adaptation stage and a 28d experimental stage. In each treatment, 10 Holstein cows in mid-lactation were selected as test animals. Cows in both the treatments were similar in parity, days in milk, and body weight (Table 1), and were maintained at the same feeding and management regime. They were kept in half-open barn equipped with feeders, water troughs, fans, and adjacent outside yards with a fixed metal framework shade.

All cows were maintained at the same management regime (milking frequency, feeding frequency, and time). They were kept in half-open barn equipped with feeders, water troughs, fans, and adjacent outside yards with a fix metal framework shade.

All cows were assigned to ten electronic gate feeders (RIC system, Insentec, Marknesse, Netherlands) with individual identification transponders fitted to each cow to record the intake of
each individual meal. Those cows were allowed free access to water and TMR daily at 0500 and 1700 h and the gate feeders were supplied with TMR to allow for 10% refusals.

The diet used in this study was formulated to meet the standard nutrient requirements of a dairy cow weighing 550 kg and producing 25 kg of milk (3.1% milkfat) daily as recommended by the National Research Council (NRC) 2001[17]. And the NEL is 6.36MJ/Kg which can meet the cow’s needs for thermal neutral conditions. The ingredients and chemical composition of the TMR was presented in Table 2.

The cows were milked twice in a day (0500 and 1700 h) in a conventional milking parlor with a machine milking system (DeLaval, Tumba, Sweden) and milk yield of the individual cow was recorded at each milking.

2.2. Sampling and Analysis

Feed samples were taken weekly and stored at -20°C until they were dried in a forced-air oven at 65°C for at least 24 h. All the feed samples were ground through a 1-mm screen and were analyzed for dry matter, ash, calcium, phosphorus, crude protein (CP), neutral detergent fiber (NDF), acid detergent fiber (ADF). Proximate analysis was performed according to AOAC [16], and fiber was analyzed according to Van Soest, et al. [18].

Milk samples (each of 25 ml volume) from each cow were collected in the morning and afternoon each Tuesday during experiment stages and composited at 5:5 as one day sample. An aliquot of one day sample of each cow was stored at 4°C before analyzing for milk components (fat, lactose, and protein) and somatic cells by Arizona DHIA (Tempe, AZ). On the last day of the experiment, the one-day milk samples (10ml) from each cow was collected and was kept cold in ice until they were defatted by centrifugation at 3,000 g for 15 min at 4°C. The skimmed milk was stored at -80°C until delivery to the laboratory for NMR analysis.

Blood samples were taken from the coccygeal vein of each cow before morning feeding on the last day of the experiment. Blood was collected in evacuated tubes containing EDTA for anticoagulation. Samples were kept on ice until they were centrifuged at 3,000 g for 10 min at 4°C. Plasma was aliquoted and stored at -80°C until NMR analysis.

2.3. Measurements

Milk production was recorded weekly. Milk yield and milk fat percentage were used to calculate 4% fat-corrected milk (FCM). Rectal temperature was determined 3 times per day using a clinical thermometer. Respiratory rate was measured at 0700, 1400 and 2200h on Monday, Wednesday, and Friday of each experimental stage by counting the number of flank movements in 1 minute and recorded as breaths per minute. Dry matter intake (DMI) was recorded daily and measured by subtracting daily refusals from daily offers to each cow.

According to NRC 2001 [17], the temperature-humidity index (THI) was calculated as (Tdb + Twb) × 0.72 + 40.6. Where Tdb is the dry-bulb temperature (°C) and Twb is the wet-bulb temperature (°C). The Tdb and Twb temperature was recorded daily at 0700h, 1400h, 2200h; Cows were weighed for 3 consecutive days before morning feeding at the beginning and end of the experiment.

2.4. 1H NMR Spectroscopic Measurement

Detailed procedures for sample preparation and NMR experiments were referred to published literature [19]. Plasma samples were thawed at room temperature and homogenized using a vortex mixer. Then 400μL of each plasma sample was placed in a 1.5mL tube and mixed with 170μL of D2O for looking at the signal and 30μL of PBS to minimize variations in sample pH. After centrifugation at 12000 rpm for 10min at 4°C, 500μL of the supernatants was transferred into 5-mm NMR tubes and stored at 4°C until analysis. The 1H NMR spectra for all specimens were acquired at 298k on a Bruker Avance 600 (Bruker BioSpin GmbH, Karlsruhe, Germany) with a 599.91Hz, an acquisition time of
One-dimensional (1D) spectra were recorded using the Carr-Purcell-Meiboom-Gill (CPMG) to suppress water signals and broad protein resonances. All 1HNMR spectra were manually phased and baseline was corrected by using MesReNova 7.1 software. Prior to Fourier transformation, the free induction decays (FIDs) were multiplied by an exponential function with a 0.3 Hz line-broadening factor. The 1HNMR spectra were referenced to the Lactate resonance at 1.33ppm. Each spectrum (0.5-9.0 ppm) was divided into 0.002ppm bins excluding the residual water region from 5.2 to 4.5 ppm. The remaining bins of each spectrum were normalized to a total spectral area of unity prior to pattern recognition.

2.5. 1HNMR Data Handling

The free induction decays were multiplied by an exponential line-broadening factor of 1.0 Hz before Fourier transformation and were manually phased, calibrated, and baseline-corrected by using MestReNova NMR software (Version 7.1). Chemical shift of each milk sample was referenced to the signal of the sodiumtrimethylsilyl-[2, 2, 3, 3-2H4]-1-propanoate (TSP) (δ 0 ppm), and chemical shift of each plasma sample was referenced to the signal of L-lactate (δ 1.33 ppm). The NMR spectra of milk samples (δ 0.6-9.0 ppm) and plasma samples (δ 0.5-9.0 ppm) were subdivided into 0.002 ppm integral regions and integrated. The part of δ 4.73-5.16 ppm and δ 3.33-3.34 ppm were removed to eliminate the base-line effects of imperfect water supersession and effects of ethanol for milk sample, and the region of δ 4.5-5.2 ppm was removed to eliminate baseline effects of imperfect water supersession for plasma sample. After exclusion of the water and ethanol resonances, each integral region was normalized to the total integral part before multivariate analysis.

Multivariate analysis, including unsupervised principal component analysis (PCA) and supervised orthogonal partial least squares discriminant analysis (OPLS-DA) were performed using SIMCA-P11.0 software (Umetrics, Umea, Sweden). PCA displays the internal structure of datasets in an unbiased way and decreases the dimensionality of data, and was used to visualize global clustering and differences in metabolic profiles between samples, and data of mean center scaling was employed for PCA. For regression, partial least squares discrimination analysis (PLS-DA) was used. The model quality was evaluated using the R^2X, R^2Y, and Q^2 parameters. The R^2X and R^2Y parameters, which represent the fractions of explained X- and Y-variations, respectively can be used to evaluate the quality of the model. The Q^2 parameter represents the predictive ability of the model. In general, the model is acceptable when the values of R^2X, R^2Y, and Q^2 are more significant than 0.5 [20]. The corresponding chemical shift and multiplicity of all metabolites were based on published spectral data and databases such as the bovine metabolome database (http://www.cowmetdb.ca/cgi-bin/browse.cgi), KEGG (http://www.genome.jp/kegg/).

After an initial overview of PCA and PLS-DA analysis, we obtained a more sophisticated OPLS-DA model with the specific discriminant information between the HS and TN period. The differences in the metabolites between groups were shown as the coefficient of variation plots. The OPLS-DA model was carried out to maximize the separation between samples by removing the variation in X matrix unrelated to Y matrix [21]. The multivariate models were validated by a 6-round cross-validation procedure to guard against over-fitting and by permutation tests (n=200). The color-coded coefficient loading plots of the OPLS-DA model were used to determine the difference between HS and TN period.

Based on the number of samples in the current study, the correlation coefficient of |r| > 0.63 and |r| > 0.60 was used as the cut-off values for significance of milk and plasma samples, respectively.

2.5. Identification of Differential Metabolites and Metabolic Pathway Analysis

As described above, PLS-DA and OPLS-DA was employed to identify differential metabolites. Then, receiver operating characteristic (ROC) curves analysis was performed (SPSS 16.0) and the areas under the curves (AUCs) were calculated to determine the diagnostic value of these differential metabolites. The KEGG was utilized to search for the metabolite pathways and enrichment analysis.
A free and web-based tool, MetaboAnalyst3.0, which uses high-quality KEGG metabolic pathway as the backend knowledge base, was used for the pathway analysis (http://www.metaboanalyst.ca).

2.6. Calculation and Statistical Analysis

The effects of heat stress on physiological parameters and milk yield were analyzed by repeated measures using the PROC GLM procedure of SAS (SAS 9.0). Least square means were separated with the PDIF procedure of SAS (SAS 9.0). Significance was declared at P<0.05.

3. Results

3.1. Environmental conditions

The THI values calculated during the experimental periods were shown in Figure 1. The mean daily THI ranged from 55.1 to 63.0 during the spring period (averaged 58.8 in the whole period) and from 75.0 to 85.8 during the summer period. The mean daily THI in the entire summer period exceeded 72, indicating that dairy cows suffered heat stress.

3.2. Physiological parameters and animal performance

Physiological parameters and performance of cows were illustrated in Table 3. Bodyweight did not differ both at the beginning and the end of HS and TN periods, but bodyweight change during the two periods varied greatly (P<0.01). Respiratory rate and rectal temperature were greater (P<0.01) in the HS period than those in the TN period.

Heat stress reduced milk yield (P<0.01), milk protein (P<0.05), protein yield (P<0.05) and DMI (P<0.05), compared with TN period. Heat stress had no effect on milk lactose content and milk fat content (P > 0.10), but milk fat yield and lactose yield were decreased compared with TN period, likely due to the overall reduction in milk yield.

3.3. 1H NMR Spectra of milk and plasma samples

The representative 600MHz 1H NMR spectra gave an overview of the metabolic profiles from milk and plasma (Figure S1 and Figure S2) of cows in HS period and TN period. Part of the assignments was listed in figure captions. By visual inspection of the 1H NMR spectra, different metabolite patterns were observed between cows in HS period and those in TN period. Milk concentrations of pyridoxamine and scylloinositol and plasma concentrations of glucose and formate appeared to be greater in cows in the HS period than in the TN period. In order to obtain more intensive analysis of metabolic differences between the two periods, we further analyzed the NMR data by using multivariate analysis including PCA, PLS-DA, and OPLS-DA to reveal the significant differences of identified metabolites between cows in HS and TN period.

3.4. Identification of Different Metabolites

The PCA and PLS-DA of the 1H NMR data from milk and plasma showed a separation between the cows in HS and TN period (Figure 2, Figure S3 and Figure S4). Further analysis using OPLS-DA revealed that concentrations of 8 metabolites in milk and 12 metabolites in plasma are significantly different between cows in HS and TN period (Figure 3 and Figure 4). The cows in the HS period had higher concentrations of NAG, choline, scyllo-inositol, pyridoxamine, and lower concentrations of OAG, citrate, GPC, methyl phosphate in their milk, than cows in TN period. The cows in HS period had greater concentrations of alanine, glutamate, glucose, urea, histidine, 1-methylhistidine, formate, and lower concentrations of VLDL, LDL, lipid, leucine, 3-hydroxybutyrate in their plasma than cows in TN period (Table 4).

In order to determine the diagnostic values of these different metabolites for differentiating of
TN from HS, ROC analysis was carried out (Figure 5 and Figure 6). Most of the different metabolites displayed excellent diagnostic abilities with AUC values of 0.70-1.00.

### 3.5.5 Integration of Key Different Metabolic Pathways

Functional pathway analysis facilitating further biological interpretation revealed the most relevant pathways. Twelve pathways were obtained; these metabolites were involved in multiple biochemical pathways, such as lipolysis, glycolysis, gluconeogenesis, amino acid metabolism, galactose, and inositol phosphate metabolism, muscle protein catabolism, purine metabolism, and glutamate metabolism (Figure 7 A and Figure 7 B). Finally, we combined these results to draw a metabolic network map (Figure 8) to show the biological significance of the variation of plasma and milk concentrations of metabolites in the heat stress period.

# 4. Discussion

In HS period, DMI dramatically decreased, and therefore cannot meet energy demand for maintenance and lactation, which is the main cause of negative energy balance, as reflected in body weight changes (Table 3). In order to compensate for the energy demands, the processes of both lipolysis and proteolysis are reinforced. The 3-methylhistidine is a product of the posttranslational methylation of histidine in both actin and myosin. Catabolism of the muscle proteins (actin and myosin) leads to the release of 3-methylhistidine (3-MH). The plasma concentration of 3-MH is a good indicator for protein mobilization in cows because it is not further metabolized in the body [22]. It is interesting that we did not find the changes in the plasma concentration of 3-MH. Instead, we found the up-regulation of 1-MH in the plasma of heat-stressed dairy cows. The 1-MH is the degradation product of anserine (β-alanyl-1-methyl-histidine) which is a dipeptide found in the muscles of many animals including cows [23]. The 1-MH is used as a biomarker for meat intake in humans because anserine is not contained in human muscle [24]. This methylation product of histidine cannot be synthesized in the human body unless anserine-containing meat is consumed. Our results indicated that anserine in cow muscle might breakdown and thus 1-MH was released during heat stress, resulting in the increased plasma concentration of 1-MH. Therefore, the plasma concentration of 1-MH may be a potential biomarker for monitoring protein mobilization in dairy cows.

Rates of protein synthesis in normal and pathologic conditions are more closely related to the intracellular amino acid pool than to plasma amino acid levels. Increased concentration of glutamate, alanine, and histidine were detected in the plasma samples from the HS group compared with those from the TN group. These amino acids may be the main precursors for glucose production via gluconeogenesis or energy production through deamination and oxidation [25]. The results are consistent with the reports of Guo et al. [26] who reported that the amino acids (Ala, Glu, Gly, and Asp) for gluconeogenesis were higher in heat-stressed cows.

Alanine synthesis occurs at a critical branch point between aerobic and anaerobic metabolism. Alanine offers at least two advantages under oxygen-limiting conditions: the conservation of carbon skeletons and the reduction in pyruvate availability for lactate and acetaldehyde synthesis. Under hypoxic stress, the synthesis of alanine increased and accumulated as the major amino acid [27]. Alanine is synthesized in muscle by transamination of pyruvate and then released into the bloodstream. The previous study has reported that Ala can regulate gluconeogenesis and glycolysis to ensure glucose production during periods of food deprivation [28]. We found higher Ala concentration in heat-stressed dairy cows, indicating that gluconeogenesis was likely reinforced under heat stress and therefore high plasma alanine concentration is also a sign of inadequate cellular energy substrates of dairy cow in heat stress period. In addition, a high concentration of glutamate via Glutamate dehydrogenase benefited for syntheses of alanine [29, 30].

Glutamate is of vital importance in both carbohydrate and amino acid metabolism. Glutamate acts as precursors for the synthesis of glucose by gluconeogenesis [31]. The metabolism of many AA, including arginine, ornithine glutamine, proline, and histidine, converges on glutamate itself [32].

Moreover, under cell stressful conditions, glutamate could be used for neurotransmitter synthesis and glutathione production to protect cells from damage fo redox crisis [33]. The increasing
glutamate synthesis is often involved in adaptive strategy for organisms to stressors. The elevated glutamate was observed in the HS group, highlighting the involvement of glutamate in multiple metabolic processes under HS. The excess amino acids accumulated in plasma indicate that the capacity of amino acid catabolism is likely reinforced in the liver [34]. These amino acids can further degrade to produce 2-oxoglutarate. Through the tricarboxylic acid cycle (TCA), 2-oxoglutarate is converted to oxaloacetic acid, which is used to synthesize glucose to supplement the energy demand. Deduced from the recent discussion; we concluded that dairy cows subjected to heat stress are suffer from protein catabolism and lack of energy substrates.

Formate is an essential endogenous one-carbon metabolite in animals participating in a vital one-carbon pool of intermediary metabolism, and was proposed as a valuable biomarker of impaired one-carbon metabolism. The endogenous source of formate is supplied by rumen methanogens which use microbial fermentation to produce products such as formic acid and hydrogen for reduction to produce methane. Therefore, there is little hydrogen and formic acid in the rumen [35].

Our result showed that plasma concentrations of formate in heat-stressed cows were up-regulated that suggested heat stress leads to impaired methanogenesis, which leads to high levels of formate entering blood. To our knowledge formate is toxic to animals. As it was already reported that formate was responsible for the toxicity observed in methanol poisoning [36]. The toxicity of formate includes optic nerve damage and metabolic acidosis [37]. Hovda, et al. [38] demonstrated that metabolic acidosis happened only at higher concentrations of plasma formate. Our result showed that the impaired one-carbon metabolism and the possible signs of metabolic acidosis of the cows during HS period.

Plasma concentration of glucose increased in HS period in this study that is in line with the findings of Srikananda Kumar and Johnso in Holstein and Australian Zebu cows [39]. But there were some contradictory reports also [6, 40]. There were evidences that both the absorption of glucose from the intestine and kidney and the production of hepatic glucose were increased during heat stress [41-43], which supports our finding of increased plasma glucose concentration in heat-stressed cow. Plasma concentration of glucose may not be a useful biomarker for dairy cows undertaking heat stress.

Significantly the decreased levels of 3-hydroxybutyrate (3-HB) were observed in the HS period. The 3-HB, the main ketone body is generated from the lipolysis in the liver mitochondria and can be used as an energy source when the supply of blood glucose is insufficient [44]. Therefore, the decreased 3-hydroxybutyrate plasma level of in heat-stressed cows might be indicated that the inhibition of β-oxidation of fatty acid was involved in the pathogenesis of heat stress [45]. The up-regulation of plasma concentration of glucose in heat stress period in this study supported the conclusion that β-oxidation of fatty acid is inhibited in heat-stressed cows. Geraert, et al. [46] demonstrated that the activities of lipolytic enzymes in monogastric animals in heat stress decreased. It was hypothesized that the limited fat mobilization during heat stress is an adaptation process to limit heat generation, and therefore, lipolysis may not be an energy-generating process for dairy cows during HS [47, 48]. But evidences are needed.

In addition, Plasma 3-HB is derived not only from liver fatty acid metabolism but also from rumen butyrate [49]. A decrease of DMI from heat-stressed cow may be associated with a decrease in plasma 3-hydroxybutyrate. Anyhow, the decreased plasma 3-HB in this study might suggest the inhibitory effect of heat stress on lipolysis in dairy cows. Plasma 3-HB level may be used as a biomarker for evaluating the susceptibility to heat stress.

A significantly decreased level of leucine was observed in cows in the HS period compared to those in the TN period. As an essential amino acid leucine has the capacity to stimulate muscle protein synthesis [50]. Although, it can be synthesized by certain anaerobic ruminal bacteria by use of pathway different from that described in other microorganisms [51]. It was believed that the decreased content of leucine was related to lower food intake and the body weights losses of heat-stressed cows. Fried, et al. [52] concluded that increased level of leucine in blood led to an increase in protein turnover in a mouse model. A decreased leucine concentration in blood plasma after a long duration of exercise may cause physical and mental fatigue [53]. Leucine is also implicated in different disease conditions. For example, Maple Syrup Urine Disease is characterized by the lack of branched-chain alpha-keto acid dehydrogenase complex [54]. The role of leucine metabolism in heart
disease is also recently reviewed [55]. Taegtmeyer, et al. [56] proposed that the supplementation of
leucine aided in the repair of heart during ischemic injury. Therefore, it may be hypothesized that the
decreased level of Leucine observed in our study may indicate the mental fatigue and ischemic injury
of the heart of dairy cows during heat stress.

Although in cattle the concentration of VLDL in plasma is extremely low, they appear to be
essential as a primary source of fats for extrahepatic tissues [57]. Our result suggested that the plasma
concentration of VLDL decreased in HS period, which is in agreement with the findings of Tian, et al.
[11]. The VLDL particles are assembled and secreted by hepatocytes [58]. The inability to rapidly
increase VLDL production may be due to deficient synthesis of the essential components, cholesterol,
phospholipids (particularly GPC) or apolipoproteins. GPC is the major phospholipid component of
VLDL, comprising approx. 60% of the total phospholipid [59]. we observed that the level of GPC
decreased but the concentration of choline was upgraded in milk form the HS group. The result might
indicate that heat stress causes abnormalities in liver lipid metabolism. A reduced ratio of GPC to
choline implies a metabolic change from Phosphatidylcholine and GPC to choline during HS period.
Decreased GPC levels in milk are favorable for the release of choline and adaptation to hot weather
conditions.

Except for a beneficial nutrient, choline was found to be positively associated with well
coaulation parameters [60]. The high choline content together with the decreased citrate content in
the dairy cow in HS period could imply an improved milk coagulation property during heat stress
because Sundekilde, et al. [60] confirmed that citrate was negatively associated with well coagulation
parameters of milk.

Protein glycosylation is a common way of protein modification and is linked to protein functions
such as protein folding, biological recognition, and enzymatic protection [61]. Glycoprotein typically
contains N-acetylactosamine units, which are built on an N- or O-linked core structure. These core
structures are the carriers of terminal carbohydrate epitopes that often confer a specific biological
property on the glycoprotein [62].

It is believed that almost all of the key molecules involved in the innate and adaptive immune
response are glycoproteins and the majority of circulating glycoproteins are acute phase reactants
and immunologic proteins. Many studies reported that heat stress impairs cow’s immune function[63,
64] and leads to a greater disease incidence in the postpartum period [65]. Heat stress also has adverse
effects on the offspring [66]. Nardone, et al. [67] found that high air temperature during late
pregnancy decreases the concentration of IgG and IgA and the percentage of total protein in the
colostrum from the first milking of primiparous dairy cows. And this will impair colostral antibody
absorption and increased mortality in the neonatal calf [68].

So far, however, no glycan-dependent function has been reported for the glycoproteins present
in cow milk. Therefore, in our study, the increased milk concentration of NAG and the decreased
milk concentration of OAG from the heat-stressed cows may be associated with impaired the cow’s
immune function and incorrect glycosylation of immunoglobulins by mammary gland epithelial cells.
So, both NAG and OAG may be used as useful milk biomarkers for heat stress in dairy cows.

Scyllo-inositol is an isomer of myo-inositol which can be synthesized in vivo from glucose-6-
phosphate [69]. As a galactose metabolism product; part of serum inositol comes from dietary phytate
degradation by certain species of intestinal bacteria [70]. Milk scyllo-inositol may either be up taken
from blood or synthesized by mammary gland epithelial cells. Scyllo-inositol was reported to reverse
memory deficit in transgenic mice with an Alzheimer’s phenotype [71]. Heat stress was reported to
stimulate the phosphoinositide turnover in HA1-CHO and BLAB/C cells, resulting in the rapid
accumulation of inositol trisphosphate (IP3). Heat shock also caused a rapid increase in
phosphorylation of polyphosphoinositides (PPI). Prolonged exposure to heat led to progressive
cellular toxicity which was associated with depletion of PPI. This decline in PPI concentration
appeared to result from inhibition of PPI resynthesize [72]. The content of scy-inositol was
upregulated by heat stress, which indicated that heat stress leads to inositol metabolism disorder and
cellular toxicity of mammary gland epithelial cells.

Pyridoxamine is one form of vitamin B6 serving as a coenzyme in various enzymatic reactions,
such as transamination and decarboxylation reactions [73]. Vitamin B6 deficiency can affect various
processes of the body such as inflammation and renal function [74]. Milk pyridoxamine, therefore
important functional nutrients beneficial to animal health. But the biological significance and the mechanism of the elevated milk concentration of pyridoxamine during HS period is not known.

Citrate is known to be involved in TCA, and it has been used as a biomarker of energy balance in dairy cows and is correlated with the presence of ketone bodies in milk and de novo fatty acids synthesis [75]. There is strong evidence that citrate in milk originates from citrate formed within mammary secretory cell rather than from that in blood plasma [76, 77]. Additionally, milk citrate concentration is an expression of mammary activity rather than energy metabolism [78]. Because mammary epithelium is impermeable to citrate in both directions [77]. Moreover, Tian’s study further confirms the hypothesis that the milk citrate levels reflect an HS-induced disturbance in mammary function rather than alterations in the blood citrate metabolism [11]. The level of citrate in milk decreased significantly during HS period, which could be a reflection of decreased mammary activity of heat-stressed dairy cows, rather than of imbalanced energy metabolism. Therefore, our result may also indicate that the de novo synthesis of fatty acids was increased in heat stress period.

5. Conclusions

In this study, the metabolites in milk and plasma of dairy cows were influenced by heat stress. The 8 metabolites in milk and 12 metabolites in plasma were altered and were mainly involved in gluconeogenesis, protein degradation and synthesis, and milk fat synthesis. These metabolites in milk and plasma could be potential biomarkers for HS. In addition, this work found several metabolites (especially in milk) which have been rarely studied. Further studies are needed to investigate the biological significance of these changes.

Author Contributions: Shuangming Yue, Siyan Ding, and Bai Xue designed the studies and prepared the manuscript with comments from all authors. Siyan Ding, Xiaofei Hu, Xiaonian Zhao, haiqian Xiong performed all the experiments and analyzed the data. Juncai Chen, Zhisheng Wang, Lizhi Wang and Quanhui Peng revised the manuscript.

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Conflicts of Interest: All authors declare that they have no competing interest in the present work.

References


**Table 1. Basic Information of the Experiment Cows**

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<tr>
<th>Periods</th>
<th>Parity</th>
<th>Days in milk</th>
<th>Body weight</th>
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<tr>
<td>Thermo-neutral period</td>
<td>3</td>
<td>146 ± 39</td>
<td>604.44 ± 56.44</td>
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<td>Heat stress period</td>
<td>3</td>
<td>145 ± 25</td>
<td>601.81 ± 78.97</td>
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**Table 2. Composition (% of DM) and Nutritional Value of Diet**

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<th>Ingredient</th>
<th>%</th>
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<tr>
<td>sweet potato vine</td>
<td>13.95</td>
</tr>
<tr>
<td>Corn meal</td>
<td>23.50</td>
</tr>
<tr>
<td>Wheat bran</td>
<td>6.00</td>
</tr>
<tr>
<td>Soybean meal</td>
<td>9.70</td>
</tr>
<tr>
<td>Rapeseed meal</td>
<td>6.00</td>
</tr>
<tr>
<td>Calcium Fatty Acids2</td>
<td>1.00</td>
</tr>
<tr>
<td>Limestone</td>
<td>1.20</td>
</tr>
<tr>
<td>Calcium superphosphate</td>
<td>1.00</td>
</tr>
<tr>
<td>Item</td>
<td>Thermo-neutral period</td>
</tr>
<tr>
<td>---------------------------</td>
<td>-----------------------</td>
</tr>
<tr>
<td>Respiration rate (breaths/min)</td>
<td>35.4 ±4.7</td>
</tr>
<tr>
<td>Rectal temperature (°C)</td>
<td>38.6 ±0.1</td>
</tr>
<tr>
<td>Initial BW (kg)</td>
<td>604.4 ±56.4</td>
</tr>
<tr>
<td>Final BW (kg)</td>
<td>612.2 ±55.6</td>
</tr>
<tr>
<td>BW changes (kg)</td>
<td>7.8 ±8.3</td>
</tr>
<tr>
<td>DMI (kg)</td>
<td>18.1 ±2.5</td>
</tr>
</tbody>
</table>

1Hemarthria altissima and sweet potato vine fresh mowing.
2Calcium Fatty Acid was produced in IFFCO (Malaysia) SDN.BND.
3Premix contains (Per kilogram of premix): VA 500000IU, VD 150000IU, VE 3000IU, Fe 4.0g, Cu 1.3g, Mn3.0g, Zn 6.0g, I 80mg, Se 50mg, Co 80mg.
4Net energy for lactation (NEL) was the calculated value, others were measured values.
<table>
<thead>
<tr>
<th>Metabolites</th>
<th>Identification (ppm) and multiplicity</th>
<th>Correlation coefficients (r)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Milk yield (kg/d)</strong></td>
<td>28.6 ±1.4</td>
<td>19.9 ±2.1</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Protein (%)</td>
<td>3.2 ±0.2</td>
<td>2.9 ±0.9</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td><strong>Protein yield (g/d)</strong></td>
<td>914.5 ±2.2</td>
<td>578.1 ±1.8</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Fat (%)</td>
<td>3.6 ±0.7</td>
<td>3.8 ±0.6</td>
<td>0.12</td>
</tr>
<tr>
<td><strong>Fat yield (g/d)</strong></td>
<td>1026.6 ±3.2</td>
<td>753.8 ±2.5</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Lactose (%)</td>
<td>4.9 ±0.2</td>
<td>4.7 ±0.2</td>
<td>0.19</td>
</tr>
<tr>
<td><strong>Lactose yield (g/d)</strong></td>
<td>1407.2 ±5.2</td>
<td>933.5 ±3.8</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

1BW=body weight, DMI= dry matter intake

**Table 4.** OPLS-DA Coefficients Derived From the NMR Data of Metabolites in Milk and plasma from Dairy Cows in TN Period and HS Period

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>Identification (ppm) and multiplicity</th>
<th>Correlation coefficients (r)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-acetyl glycoprotein</td>
<td>2.06(s)</td>
<td>-0.73</td>
<td>0.02</td>
</tr>
<tr>
<td>O-acetyl glycoprotein</td>
<td>2.07(s)</td>
<td>0.68</td>
<td>0.01</td>
</tr>
<tr>
<td>Citrate</td>
<td>2.53(d), 2.67(d)</td>
<td>0.74</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Choline</td>
<td>3.20(s)</td>
<td>-0.64</td>
<td>0.03</td>
</tr>
<tr>
<td>Glycerophosphorylcholine</td>
<td>3.23(s)</td>
<td>0.63</td>
<td>0.01</td>
</tr>
<tr>
<td>Methyl phosphate</td>
<td>3.49(d)</td>
<td>0.68</td>
<td>0.1</td>
</tr>
<tr>
<td>Scyllo-inositol</td>
<td>3.36(s)</td>
<td>-0.66</td>
<td>0.02</td>
</tr>
<tr>
<td>Pyridoxamine</td>
<td>7.67(s)</td>
<td>-0.68</td>
<td>0.02</td>
</tr>
<tr>
<td>L2, L4: VLDL</td>
<td>0.89(br), 1.29(br)</td>
<td>0.81</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>L3: LDL</td>
<td>1.27(br)</td>
<td>0.66</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>L6: Lipid</td>
<td>2.01(br)</td>
<td>0.64</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Leucine</td>
<td>0.96(t), 1.69(m)</td>
<td>0.75</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>3-Hydroxybutyrate</td>
<td>1.20(d)</td>
<td>0.74</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Alanine</td>
<td>1.48(d)</td>
<td>-0.64</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Glutamate</td>
<td>2.13(m), 2.46(m)</td>
<td>-0.65</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Glucose</td>
<td>3.42(t), 3.54(dd), 3.71(t), 3.73(m), 3.84(m), 5.23(d)</td>
<td>-0.71</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Urea</td>
<td>5.78(br)</td>
<td>-0.66</td>
<td>0.02</td>
</tr>
<tr>
<td>Histidine</td>
<td>7.05(s), 7.76(s)</td>
<td>-0.63</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>1-Methylhistidine</td>
<td>6.96(s), 7.61(s)</td>
<td>-7.64</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Formate</td>
<td>8.45(s)</td>
<td>-0.68</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

1VLDL = very low density lipoprotein; LDL = low density lipoprotein.

2Multiplicity: s = singlet, d = doublet, t = triplet, q = quartet, dd = doublet of doublets, m = multiplet, br = broad resonance.

3 Correlation coefficients, Positive and negative signs indicate positive and negative correlation in the concentrations of milk metabolites in dairy cows in TN group relatively to cows in HS group. The correlation coefficient of | r | > 0.602 was used as the cutoff value for the statistical significance based on the discrimination significance at the level of p=0.05 and df (degree of freedom) = 9.
Correlation coefficients, positive and negative signs indicate positive and negative correlation in the concentrations, respectively. The correlation coefficient of $|r| > 0.553$ was used as the cutoff value for the statistical significance based on the discrimination significance at the level of $p=0.05$ and $df$ (degree of freedom) = 9.

$^3$P-value of independent of t-test for HS verses TN.

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**Figure 1.** The values of temperature-humidity index (THI) (mean /d) during summer period (n=10, triangle line) and spring period (n=10, rectangle line).

**Figure 2.** PCA scores plot based on $^1$H NMR spectra of aqueous phase obtained from HS and TN group (left). PCA scores plot based on $^1$H CPMG NMR spectra of plasma obtained from HS and TN group (right). A=Heat Stress group ;B=Thermal neutral group
Figure 3. OPLS-DA scores plots (left panel) and corresponding coefficient loading plots (right panel) of milk. A=Heat Stress group ;B=Thermal neutral group

Figure 4. OPLS-DA scores plots (left panel) derived from $^1$H NMR spectra of plasma and corresponding coefficient loading plots (right panel) obtained from different pair-wise groups. A=Heat Stress group ;B=Thermal neutral group
Figure 5. ROC analysis of bio-markers from milk.

ROC Curve

Sensitivity

1-Specificity

1.0
0.8
0.6
0.4
0.2
0.0

0
0.2
0.4
0.6
0.8
1

NAG
OAG
Citrate
Choline
PC
GPC
MP
scyllo-inositol
Pyridoxamine
Reference line
Figure 6. ROC analysis of bio-markers from plasma for cows in HS and TN period
Figure 7. The metabolome view map of significant metabolic pathways characterized in milk (A) and plasma (B) for cows in HS and TN period. This figure aims to find pathways significant changed based on enrichment and topology analysis. The x-axis represents pathway enrichment, and the y-axis represents pathway impact. Larger sizes and darker colors represent greater pathway enrichment and higher pathway impact values, respectively.
Figure 8. Metabolic network of the potential bio-markers in plasma and milk that changed between the HS and TN period. The increased metabolites in the HS period are shown in red. The decreased metabolites in the HS period are shown in blue.

Supplementary material:
Figure S1. A representative 600 MHz 1H NMR spectra (δ0.5-5.0 and δ5.0-9.5) of milk from HS period and TN period. The region of δ5.3-9.5 (in the dashed box) was magnified 32 times compared with corresponding region of δ0.5-5.3 for the purpose of clarity.

1-MH: 1-Methylhistidine; 3-HB: 3-Hydroxybutyrate; Crea: creatine; Glu: glutamate; GPC: glycerolphosphocholine; Ile: isoleucine; L1 and L3 lipid: LDL; L2, L4 and L5 lipid: VLDL; L6 lipid: \(-\text{CH}_2-\text{CH}=\text{CH}\); L7 lipid: \(-\text{CH}_2-\text{CH}=\text{CH}\); L8 lipid: \(-\text{CH}_2-\text{C}=\text{O}\); L9 lipid: \(=\text{CH-CH}_2-\text{CH}=\); Leu: leucine; Lys: lysine; NAG: N-acetyl glycoprotein; PC: phosphocholine; Phe: phenylalanine; Val: valine.

Figure S2. A representative 600MHz 1H NMR spectrum (δ0.5-5.5 and δ5.5-9.0) of plasma from HS period and TN period. The region of δ5.2-9.0 (in the dashed box) was magnified 16 times compared with corresponding region of δ0.5-4.6 for the purpose of clarity.

3-HB: 3-Hydroxybutyrate; GPC: glycerophosphorylcholine; Ile: isoleucine; Leu: leucine; MP: methyl phosphate; NAG: N-acetyl glycoprotein; OAG: O-acetyl glycoprotein; PC: phosphorylcholine; TMAO: trimethylamine N-oxide; Val: valine;
R²X=43.7%, R²Y=74.3%, Q²=0.242

Figure S3. PLS-DA scores plots (left panel) derived from ¹H NMR spectra of aqueous phase of milk extract. A=Heat Stress group; B=Thermal neutral group.

R²X=34.6%, R²Y=90.8%, Q²=0.641

Figure S4. PLS-DA scores plots (left panel) derived from ¹H NMR spectra of serum obtained from different groups and cross validation (right panel) by permutation test (n=300). A=Heat Stress group; B=Thermal neutral group.