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Original Research Article

Plasma metabolic profiling reveals that chromium yeast alleviates the negative effects of heat stress in mid-lactation dairy cows

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ABSTRACT

Chromium yeast (CY) supplementation has the potential to alleviate the negative effects of heat stress in dairy cows, but the mechanism remains elusive. We aimed to identify the metabolic mechanisms whereby CY supplementation alleviates the negative effects of heat stress in mid-lactation dairy cows. Twelve Holstein dairy cows with similar milk yield (24.6 ± 1.5 kg/d), parity (2 or 3) and days in milk (125 ± 8 d) were fed the same basal diet containing 0.09 mg of Cr/kg DM. They were allocated randomly to 2 groups: a control group (CON, without CY supplementation) and a CY group (CY, administered 0.36 mg Cr/kg DM). The experiment was performed over 8 weeks during a hot summer, in which the mean temperature-humidity index was 79.0 ± 3.13 (>72), indicating that the dairy cows were exposed to heat stress. Chromium yeast supplementation reduced rectal temperature ($P = 0.032$), and increased the lactation performance by increasing the yield of milk (+2.6 kg/d), protein, lactose and total solid, and protein and lactose percentages in the milk of the heat-stressed dairy cows ($P < 0.05$). Supplementation with CY increased the serum glucose and thyroxine concentrations, but reduced the urea nitrogen, insulin, and triiodothyronine concentrations on d 56 ($P < 0.05$). Furthermore, plasma metabolomic analysis was performed using liquid chromatography tandem-mass spectrometry, which identified 385 metabolites in the two groups. Subsequently, 16 significantly different metabolites in the plasma, were significantly higher in the CY group (variable importance for the projection >1.0, $P < 0.05$), and found to be involved in 6 Kyoto Encyclopedia of Genes and Genomes pathways, including those involved in nicotinate and nicotinamide metabolism. Specifically, plasma concentration of nicotinamide was higher after CY supplementation, which might also contribute to the reduction of rectal temperature, the regulation of glucose homeostasis, and an improvement in the lactation performance of heat-stressed dairy cows. In conclusion, CY supplementation reduces rectal temperature, influences metabolism by reducing serum insulin concentration and increasing serum glucose and plasma nicotinamide concentrations, and finally increases lactation performance of heat-stressed dairy cows.

1. Introduction

Heat stress is a reflex reaction in animals that are subjected to a heat load that exceeds their capacity for heat dissipation (Das et al., 2016). Heat stress is a worldwide challenge for the dairy industry because it causes economic losses during the summer. Furthermore, with the intensification of global warming, the threat posed by heat stress will become more severe (Das et al., 2016). Environment-induced hyperthermia reduces the lactation and reproductive performance of dairy cows (Collier et al., 2017; Negrón-Pérez et al., 2019), and chromic heat stress affects nutrient metabolism and hormone production (Baumgard and Rhoads, 2013; Han et al., 2019; Abbas et al., 2020). Specifically, heat stress

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directly affects glucose metabolism (Slimen et al., 2016; Abbas et al., 2020): the circulating concentration of glucose decreases in dairy cows (Settivari et al., 2007), and heat-stressed dairy cows show greater glucose consumption in tissues other than mammary glands under chronic heat stress conditions (Wheelock et al., 2010; Slimen et al., 2016). In addition, there is a downregulation of mammary protein synthesis in heat-stressed dairy cows, which results in a reduction in milk protein concentration (Cowley et al., 2015). Furthermore, heat-stressed dairy cows tend to reduce their thyroid hormone production in order to compensate for the greater metabolic stimulation and limit heat production (Magdub et al., 1982).

Trivalent chromium (Cr), an active component in glucose tolerance factor (GTF), is a micronutrient that potentiates the action of insulin (Mertz, 1993; Nishimura et al., 2021), and therefore regulates carbohydrate, lipid, and protein metabolism (Anderson, 1981). Dietary supplementation with Cr has been recommended for adult humans at a dose of 0.05 to 0.2 mg Cr/d (NRC, 1989) because it is a necessary element for glucose metabolism (NRC, 2001). Chromium increases glucose uptake by cells by improving the binding of insulin to extracellular receptors, which causes the mobilization of the insulin-dependent glucose transporter type 4 (GLUT4) (Hua et al., 2012; Vincent, 2015; Nishimura et al., 2021). Although no recommendation is drawn for the supplementation with Cr in typical cattle diets, some investigations in cattle have demonstrated a positive response to Cr supplementation, especially when the animals are under stress (Moonsie-Shageer and Mowat, 1993; Ribeiro et al., 2020; Hung et al., 2021). Heat stress leads to the loss of Cr through urinary excretion, which impairs the cellular glucose uptake (Pechova and Pavlata, 2007). Therefore, the inclusion of Cr in the diet as a means of improving glucose metabolism has become a topic of interest. Bin-Jumah et al. (2020) reviewed that Cr combats thermal stress in livestock by improving feed intake, growth rate, glucose metabolism, reproductive parameters and immune function. Accumulated data show that Cr supplementation may reduce body temperature and alleviate the impact of heat stress in dairy cows (Al-Saiady et al., 2004; Zhang et al., 2014; Shan et al., 2020).

Chromium yeast (CY) is a type of organic Cr that is obtained from yeast cells cultured in a Cr-rich medium (Toepfer et al., 1976) and is thought to be one of the best Cr carriers (Al-Saiady et al., 2004). The CY complex contains various ingredients, such as active peptides, amino acids, and B family vitamins (Shurson, 2018), and Cr is chelated by components of yeast including niacin (NA), glycine, and cystine, forming GTF, which potentiates the actions of insulin (Urumow and Wieland, 1984; Lukaski, 1999). Pantelić et al. (2018) demonstrated that CY affects the activity of the insulin signaling pathway in dairy cows, but the signaling molecules affected depends upon the energy status of the cows. Another previous study showed that CY improves the feed intake of lactating dairy cows that are under heat stress (Al-Saiady et al., 2004). Furthermore, CY has been reported to increase serum glucose concentration and reduce the concentrations of insulin and non-esterified fatty acids in heat-stressed dairy cows (Yari et al., 2010). Finally, in our previous study, we showed that CY improves the lactation performance and antioxidant and immune function of dairy cows under heat stress, and identified a suitable level of CY supplementation to be 0.36 mg Cr/kg DM (Shan et al., 2020). Thus, supplementing the diet of dairy cows with CY alleviates heat stress and its negative effects.

To the best of our knowledge, few studies have been conducted regarding the mechanisms whereby CY supplementation alleviates the effects of heat stress in dairy cows, and especially regarding the role of in vivo changes in metabolism. Therefore, the objective of the present study was to identify the metabolic mechanism whereby CY supplementation ameliorates the negative effects of heat stress in dairy cows. To this end, we determined the effects of dietary supplementation of CY on the lactation performance and serum biochemical parameters and hormones in heat-stressed dairy cows. We then used liquid chromatography tandem-mass spectrometry (LC-MS/MS) to identify significantly different metabolites in the plasma in order to determine the role of metabolic changes resulting from the effects of CY supplementation.

2. Materials and methods

2.1. Animal ethics statement

All the procedures in this study were approved by the Ethics Committee of the Chinese Academy of Agricultural Sciences (Beijing, China), and the dairy cows were cared for in accordance with the standards established by the Institute of Animal Science, Chinese Academy of Agricultural Sciences (Beijing, China).

2.2. Animals, diets and experimental design

The present study was performed between July and September 2019 on Junyuan Dairy Farm (Shijiazhuang, Hebei Province, China). The body condition and health of cows were monitored and recorded throughout the experiment. The cows were under heat stress conditions during the pre-trial and study period, with the mean temperature-humidity index (THI) of 73.4 ± 1.27 and 79.0 ± 3.13, respectively.

Twelve mid-lactation Holstein dairy cows (milk yield: 24.6 ± 1.5 kg/d, parity 2 or 3, days in milk: 125 ± 8 d) were randomly allocated to 2 groups ($n = 6$ each). All cows were fed the same total mixed ration (TMR) without CY supplementation. Table 1 shows the composition and nutritional content of the TMR used; the level of nutrients met or exceeded the National Research Council (2001) recommendations for dairy cows. The background Cr concentration in the TMR was 0.09 mg Cr/kg DM. The CY provided contained 996 mg/kg Cr (analyzed value) and was obtained from China Angel Yeast Co., Ltd (Yichang City, Hubei Province, China). The TMR was provided to each cow after milking each day (06:30, 13:30, and 20:30). The quantities of diet that were refused were approximately 5% to 10% of the total, and these quantities were removed and weighed every morning. The pre-trial period lasted for 2 weeks, during which all the cows consumed the same TMR, without CY supplementation. The experimental period was 8 weeks long, during which the experimental cows received TMR supplemented with CY at 0.36 mg Cr/kg DM. The supplemented dose of CY was based on our previous study (Shan et al., 2020). The appropriate amount of CY was mixed with 100 g of ground corn and fed with the TMR during the morning feed. All the cows were housed in a mechanically ventilated tie stall barn, separated from other dairy cows on the farm by a rail fence, divided into 2 plots with 6 cows each, and had free access to clean water and TMR.

2.3. Sampling and analysis

The ambient temperature and relative humidity were monitored daily at 06:00, 14:00 and 22:00 using a thermo-hygrometer (Beijing Yaguang Equipment Co. Ltd., Beijing, China) that was suspended in the center of the barn, 1.5 m above the ground. The THI was calculated using the published formula: $\mathrm{THI} = (1.8 \times T + 32) - [(0.55 - 0.0055 \times \mathrm{RH}) \times (1.8 \times T - 26.8)]$ (Dikmen and Hansen, 2009), where T is the ambient temperature (°C) and RH is the relative humidity (%).

The daily environment data are shown in Fig. 1A, and mean THI was used to construct a temperature and humidity index curve

Table 1
Ingredients and nutrient levels of the basal diet (% of DM).

Item	Content
Ingredients	
Corn silage	27.7
Ground corn	14.8
Ryegrass[1]	12.1
Soybean meal	9.1
Oats	7.7
Flaked corn	7.0
Sugar beet pulp	3.8
Whole cottonseed	3.1
Syrup	2.9
Distiller's dried grains	2.2
Alfalfa hay	2.1
Yeast culture[2]	1.2
Chinensis	1.1
Fat powder[3]	1.1
Limestone	1.0
$NaHCO_3$	0.7
$CaHPO_4$	0.4
NaCl	0.4
K_2CO_3	0.2
MgO	0.1
Premix[4]	1.3
Total	100.0
Nutrient levels[5]	
CP	15.90
NDF	26.09
ADF	13.75
Ca	0.83
P	0.47
Cr, mg/kg	0.09
NE_L[6], MJ/kg	6.88

[1] Ryegrass green chopped.
[2] Diamond V XP yeast culture supplement (Diamond V, USA).
[3] Bergafat, a saturated free fatty acid supplement (Berg + Schmidt, Germany).
[4] The premix provided the following per kg of the diet: vitamin A 200,000 IU, vitamin D 40,000 IU, vitamin E 5,000 IU, Mg 95,200 mg, Zn 1000 mg, Co 50 mg.
[5] Analyzed value.
[6] NE_L was a calculated value according to NRC (2001).

(Fig. 1B), in which the THI was the mean of 3 THI a day and standard deviation (SD) was the mean daily THI. A glass mercury thermometer (Nasco, Fort Atkinson, Wisconsin) was used to measure the rectal temperature (RT) of dairy cows at 07:30, 14:30, and 19:30 every day. The respiratory rate (RR) was determined 3 times daily (at 07:30, 14:30, and 21:30) by counting the number of flank movements/min.

Samples of the TMR were collected on 3 consecutive days per week during the experimental period to assay its DM content. The individual daily DMI of the cows was calculated by subtracting the mass of TMR refused from the mass offered (on a DM basis). All the samples were mixed and dried at 65 °C for 48 h, then ground such that they could pass through a 1-mm screen. Then standard procedures of the Association of Official Analytical Chemists were used for the analysis of DM (AOAC, 2005; method 930.15), crude protein (AOAC, 2000; method 976.05), calcium (AOAC, 1990; method 985.35), and phosphorus (AOAC, 1990; method 986.24) content. The NDF and ADF contents were measured using the methods described by Van Soest et al. (1991). The composition and nutrient levels of the TMR are listed in Table 1.

The cows were milked 3 times daily (05:30, 12:30, and 19:30), and their individual milk yields were recorded during each milking. Milk samples were collected every week from each cow over 3 consecutive milkings and combined at a 4:3:3 ratio (Ma et al., 2020). The milk samples were kept fresh by adding preservative (Bronopol Tablet, D&F Control System, San Ranmon Inc., Dublin, ON, Canada), then stored at 4 °C. The milk composition (fat, protein, lactose, SNF, and TS) were determined by infrared analysis (FOSS MilkoScan 2000, FOSS Food Technology Corp., Eden Prairie, MN, USA).

Blood samples were collected from the coccygeal of each cow into vacutainer tubes, with or without heparin sodium (BD Biosciences, San Jose, CA, USA), before the morning feeding on d 56. These samples were centrifuged (LDZ5-2, Beijing Leiboer Centrifuge Co., Ltd., China) at 3000 × *g* for 15 min at 4 °C to obtain plasma and serum samples, which were stored at −20 °C until analysis.

The concentration of Cr in the CY and TMR was determined using inductively coupled plasma mass spectrometry/vapor generation (Agilent 8800, Agilent Technologies, CA, USA), on the basis of the Chinese National Standards (GB 5009.268, China, 2016). An automated biochemical analyzer (Hitachi 7080; Hitachi Valve, Ltd., Tokyo, Japan) was used to measure the serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities, and the serum glucose, urea nitrogen, and uric acid concentrations. In addition, bovine ELISA kits from Wuhan Colorful Gene Biological Technology Co., Ltd (Wuhan, China) were used to determine the serum concentrations of insulin, glucagon (GC), cortisol (COR), prolactin (PRL), triiodothyronine (T3), and thyroxine (T4), according to the manufacturer's instructions. The intra- and interassay CV for insulin, GC, COR, PRL, T3 and T4 were 4.6% and 7.8%, 3.8% and 5.3%, 3.3% and 6.8%, 3.6% and 9.4%, 6.2% and 8.3%, and 7.0% and 9.7%, respectively.

2.4. Metabolomic analysis

Metabolomic analysis of the plasma samples was performed by liquid chromatography (LC)-mass spectrometry (MS)/MS using an UltiMate_3000 (Dionex, Sunnydale, CA, USA) coupled to a Q-Exactive mass spectrometer (Thermo Fisher Scientific, MA, Waltham, USA).

The plasma samples were thawed at 4 °C, then 100 μL of each sample was transferred to 1.5-mL Eppendorf tubes. Subsequently, 1 mL of a pre-cooled chloroform-methanol–water (1:2:1, vol:vol:vol) mixture was added to each, and after vortexing and ultrasonication, the samples were centrifuged at 4 °C and 13,000 × *g* for 15 min. The supernatants were filtered through 0.22-μm membranes and dried under liquid nitrogen, then stored at −80 °C until analysis. Subsequently, the samples were reconstituted in 100 μL of an isopropanol-acetonitrile-water (2:5:3, vol:vol:vol) mixture and the supernatant was separated for metabolomic analysis.

During analysis, the samples were placed in an autosampler at 4 °C, then separated by ultra-high performance liquid chromatography. A Waters C18 column at 45 °C and a flow rate of 0.35 mL/min were used. The mobile phases were 0.1% formic acid in H_2O (A) and acetonitrile (B). Chromatographic gradient elution was performed as follows: 0 to 0.5 min, 98% mobile phase A; 0.5 to 15 min, linear change from 98% to 2% mobile phase A; 15 to 17 min, mobile phase A remains at 2%; 17 to 20 min, linear change from 2% to 98% mobile phase A. Electrospray ionization (ESI) was used in both positive ion and negative ion detection modes and the ESI conditions are shown in Appendix Table 1.

2.5. Quality control

A quality control (QC) sample was also prepared that was a mixture of all the samples in equal volumes, and this was used to optimize the LC-MS/MS system and evaluate its stability.

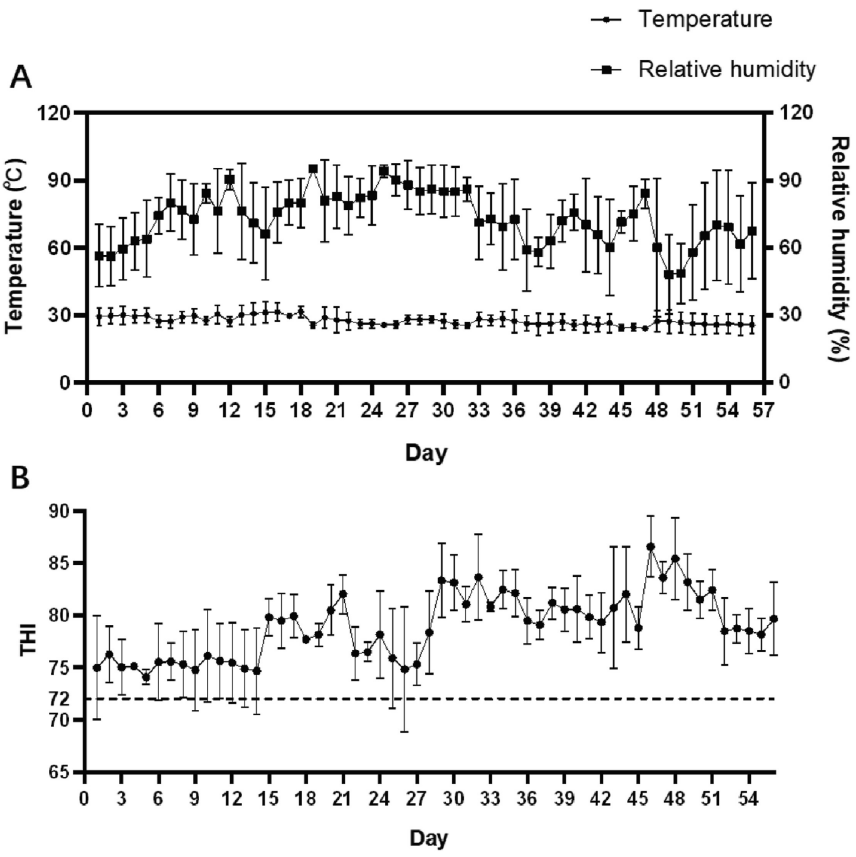

Fig. 1. The environmental data and temperature and humidity index (THI) during the study period. (A) The daily mean and standard deviation of temperature and relative humidity in the barn. (B) The daily mean THI and standard deviation of the mean daily THI in the barn.

2.6. Data processing

The raw data were processed using Compound Discoverer 3.0 (Thermo Fisher Scientific, MA, USA) for peak alignment, the correction of retention time and the measurement of peak areas. To identify metabolite structures, accurate mass matching (<25 ppm) and secondary spectrum matching methods were used to search the MZcloud database (https://www.mzcloud.org/). The data were normalized to the total peak areas, and Pareto-scaling was processed in SIMCA-P 14.1 (Umetrics, Umea, Sweden). Metabolites for which >50% of the values were missing in the raw data were not further analyzed. Multi-dimensional statistical analysis was performed, including unsupervised principal component analysis (PCA) and orthogonal partial least-squares discriminant analysis (OPLS-DA).

2.7. Identification of significantly different metabolites and pathway analyses

According to the variable importance for the projection (VIP) values obtained using the OPLS-DA model, the influence of each metabolite on the classification of each group was assessed and the explanatory power was calculated. In addition, the metabolites with potential biological significance were highlighted. Those with both a VIP >1.0 on multi-dimensional statistical analysis and a *P*-value <0.05 on univariate analysis were defined as the significantly different metabolites in the plasma of the 2 groups of cows. The significantly different metabolites were then subjected to Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.genome.jp/kegg/) pathway analysis.

2.8. Quantification of a significantly different metabolite by LC-MS/MS

Next, the concentration of nicotinamide (NAM), which was present in significantly different concentrations between the groups, was determined. A standard compound of purity >99.0% and ganciclovir (the internal standard, IS) were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The serum NAM concentration was quantified using a previously reported method (Yu et al., 2010). Briefly, 20 μL samples were injected into the system and passed through an amide column (Curosil-PFP column; 250 mm × 4.6 mm, 5 μm diameter; Phenomenex, Torrance, CA, USA) heated to 35 °C, with a flow rate of 1.0 mL/min. The mobile phases were water (A) and methanol (B), both of which contained 0.1% formic acid. The gradient conditions were the following: 0.0 min (A:B, 85%:15%) → 1.5 min (A:B, 85%:15%) → 2.0 min (A:B, 5%:95%) → 4.5 min (A:B, 5%:95%) → 4.6 min (A:B, 85%:15%) → 6.0 min (A:B, 85%:15%). Data were acquired in positive-ion ESI and selected reaction monitoring (SRM) modes. The mass spectrometry operating parameters were set as: spray voltage of 5.0 kV; heated temperature of 350 °C, and nitrogen sheath and auxiliary gases at 276 and 34 kPa, respectively. The NAM and IS were dissociated by argon collision at 0.20 Pa and 23 eV. Quantification was carried out using SRM with *m/z* values of 123.1 → 80.1 for NAM and 256.1 → 152.1 for the IS. The data were analyzed using Xcalibur

1.2 software (Thermo Fisher Scientific, MA, USA), including instrument control, data acquisition and processing.

2.9. Statistical analysis

All data were analyzed using SAS version 9.4 (SAS Institute Inc., Cary, NC, USA). Milk yield, milk composition and DMI were analyzed by unstructured covariance with repeated measurements using the Mixed procedure. Measurements of DMI, milk yield, and composition during the pre-treatment period served as covariates for the corresponding treatment period. The statistical model contained fixed effects of the study week, treatment group, and the interaction of week and treatment, and the random effect of cow identity. Serum hormone and marker concentrations, and plasma metabolite concentrations were analyzed using ANOVA in the GLM procedure of SAS. Physiological parameters were firstly calculated using the mean of each parameter every day and then statistical analysis was conducted using the same procedure as the other variables. The data are shown as least-square means and standard error of the means (SEM). Statistical significance was defined as $P \leq 0.05$ and statistical trends as $0.05 < P < 0.10$.

3. Results

3.1. Thermoregulatory responses and lactation performance

As illustrated in Fig. 1, the THI (79.0 ± 3.13) stayed above 72 throughout the 8 weeks of the experimental period, which indicates that the cows were under heat stress. Because samples of feed and milk were collected every week during the treatment period, the variables in Table 2 were tested for treatment, week, and treatment × week interactions. But week and treatment × week interaction were not significant for all variables. The mean RT and RR of the cows in the CON group were 39.6 °C and 79.3 breaths/min, respectively. Supplementation with CY significantly reduced the RT ($P = 0.032$) and tended to reduce the RR of the heat-stressed dairy cows ($P = 0.079$). This also increased the milk yield of the cows ($P = 0.031$). Furthermore, milk protein percentage and yield ($P = 0.023$ and 0.020, respectively), milk lactose percentage and yield ($P = 0.016$ and 0.001, respectively), and milk total solid yield ($P = 0.024$) were also increased by CY supplementation.

Table 2
Effects of chromium yeast on thermoregulatory responses and lactation performance of heat-stressed dairy cows.

Item	Treatment[1]		SEM	*P*-value
	CON	CY		
RT, °C	39.6	39.4	0.05	0.032
RR, breaths/min	79.3	73.2	2.24	0.079
Dry matter intake, kg/d	17.1	18.5	0.75	0.243
Yeast intake from TMR, g/d	206	221	8.8	0.243
Total yeast intake, g/d	206	228	9.0	0.116
Milk composition, %				
Protein	3.30	3.45	0.041	0.023
Fat	3.71	3.89	0.072	0.104
Lactose	4.71	5.00	0.071	0.016
Solid not fat	9.03	8.92	0.192	0.698
Total solid	13.0	13.1	0.360	0.941
Yield, kg/d				
Milk yield	23.1	25.7	0.75	0.031
Protein	0.79	0.85	0.012	0.020
Fat	0.90	0.95	0.018	0.153
Lactose	1.11	1.26	0.029	0.001
Solid not fat	2.09	2.30	0.058	0.066
Total solid	3.01	3.35	0.271	0.024

RT = rectal temperature; RR = respiratory rate; TMR = total mixed ration.
[1] CON: control group ($n = 6$, without chromium yeast supplementation); CY: chromium yeast group ($n = 6$, received additional chromium yeast at 0.36 mg Cr/kg DM).

3.2. Serum biochemical parameters and hormone concentrations

Table 3 lists the serum concentrations of biochemical parameters in each group. Supplementation with CY significantly increased the serum glucose concentrations ($P = 0.035$), but reduced the serum urea nitrogen concentrations of the cows ($P = 0.039$). In addition, the serum ALT activities tended to be lower in the CY group than in the CON group ($P = 0.067$). However, there were no other differences in serum biochemical parameters between the CY and CON groups.

Supplementation with CY significantly reduced the serum concentrations of insulin ($P = 0.044$) and T3 ($P = 0.043$), but increased that of T4 ($P = 0.032$), compared with the CON group (Table 3). In addition, it tended to increase the serum prolactin concentration ($P = 0.086$), but it had no effects on the serum glucagon and cortisol concentrations of the cows.

3.3. Plasma metabolomes

The base peak plots of the mass spectra obtained in the positive and negative ion detection modes for the QC samples were superimposed and compared (Appendix Fig. 1). The response intensity and retention time of each peak largely overlapped, which indicates that the variation caused by instrument error was small and that the data were of sufficient quality. Therefore, differences in the spectra generated were referable to genuine differences in the plasma biochemistry of the 2 groups. The PCA analysis indicates how the identified compounds discriminate the groups, and the dimensional separation ($R^2X = 0.86$ and $Q^2 = 0.204$) obtained indicates that CY supplementation influenced the plasma metabolite profile of the heat-stressed dairy cows (Fig. 2A). Furthermore, to better demonstrate the difference between the CON and CY groups, a supervised OPLS-DA model was constructed (Fig. 2B), and the evaluation parameters were $R^2Y = 0.99$ and $Q^2 = 0.897$, which show that the model was stable and capable of reliable prediction.

A total of 385 metabolites were identified in the CON and CY groups by LC-MS/MS analysis (Appendix Table 2). Of these, 16 were present at significantly different concentrations (VIP > 1.0, $P < 0.05$) between the CY and CON groups, and a heatmap showing the metabolites in the form of a hierarchical clustering analysis is shown in Fig. 3. This shows that each of the metabolites was

Table 3
Effects of chromium yeast on serum biochemical parameters and hormones in heat-stressed dairy cows.

Item	Treatment[1]		SEM	*P*-value
	CON	CY		
ALT, U/L	29.0	21.8	2.51	0.067
Aspartate aminotransferase, U/L	83.3	83.2	5.87	0.979
Glucose, mmol/L	3.39	3.75	0.102	0.035
Urea nitrogen, mmol/L	5.17	4.24	0.278	0.039
Uric acid, μmol/L	75.0	65.8	5.81	0.294
Glucagon, pg/mL	178	205	11.4	0.133
Insulin, mU/L	25.4	19.9	1.69	0.044
Cortisol, ng/mL	56.3	55.0	2.05	0.658
Prolactin, ng/mL	14.8	17.2	0.90	0.086
T3, ng/mL	691	636	16.7	0.043
T4, ng/mL	12.9	14.0	0.32	0.032
Insulin:glucose ratio	6.75	5.90	0.394	0.159

ALT = alanine aminotransferase; T3 = triiodothyronine; T4 = thyroxine.
[1] CON: control group ($n = 6$, without chromium yeast supplementation); CY: chromium yeast group ($n = 6$, received additional chromium yeast at 0.36 mg Cr/kg DM).

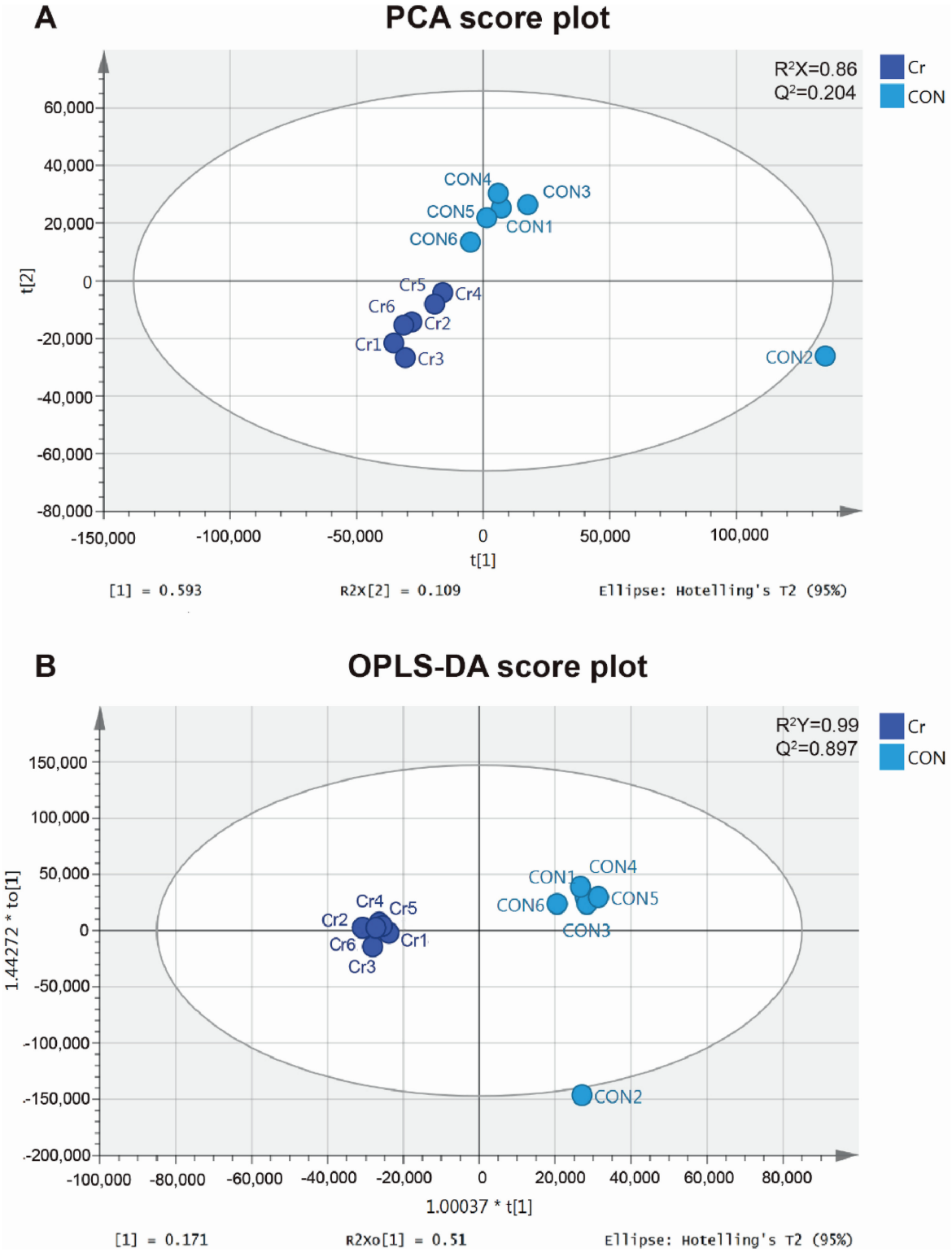

Fig. 2. The plots of principal component analysis (PCA) and orthogonal partial least-squares discriminant analysis (OPLS-DA) for plasma samples obtained from each dairy cow. (A) The PCA plots for plasma samples obtained from each dairy cow. (B) The OPLS-DA plots for plasma samples obtained from each dairy cow. CON: control group ($n = 6$, without chromium yeast supplementation); Cr: chromium yeast group ($n = 6$, received additional chromium yeast at 0.36 mg Cr/kg DM).

present in the plasma of the cows at a significantly higher concentration in the CY group than in the CON group ($P < 0.05$). These metabolites were subjected to KEGG analysis, which showed that they were involved in 6 metabolic pathways: caprolactam degradation, metabolic pathways, microbial metabolism in diverse environments, nicotinate and NAM metabolism, vitamin digestion and absorption, and drug metabolism-cytochrome P450. Ultimately, 4 KEGG pathways with $P < 0.05$ were enriched:

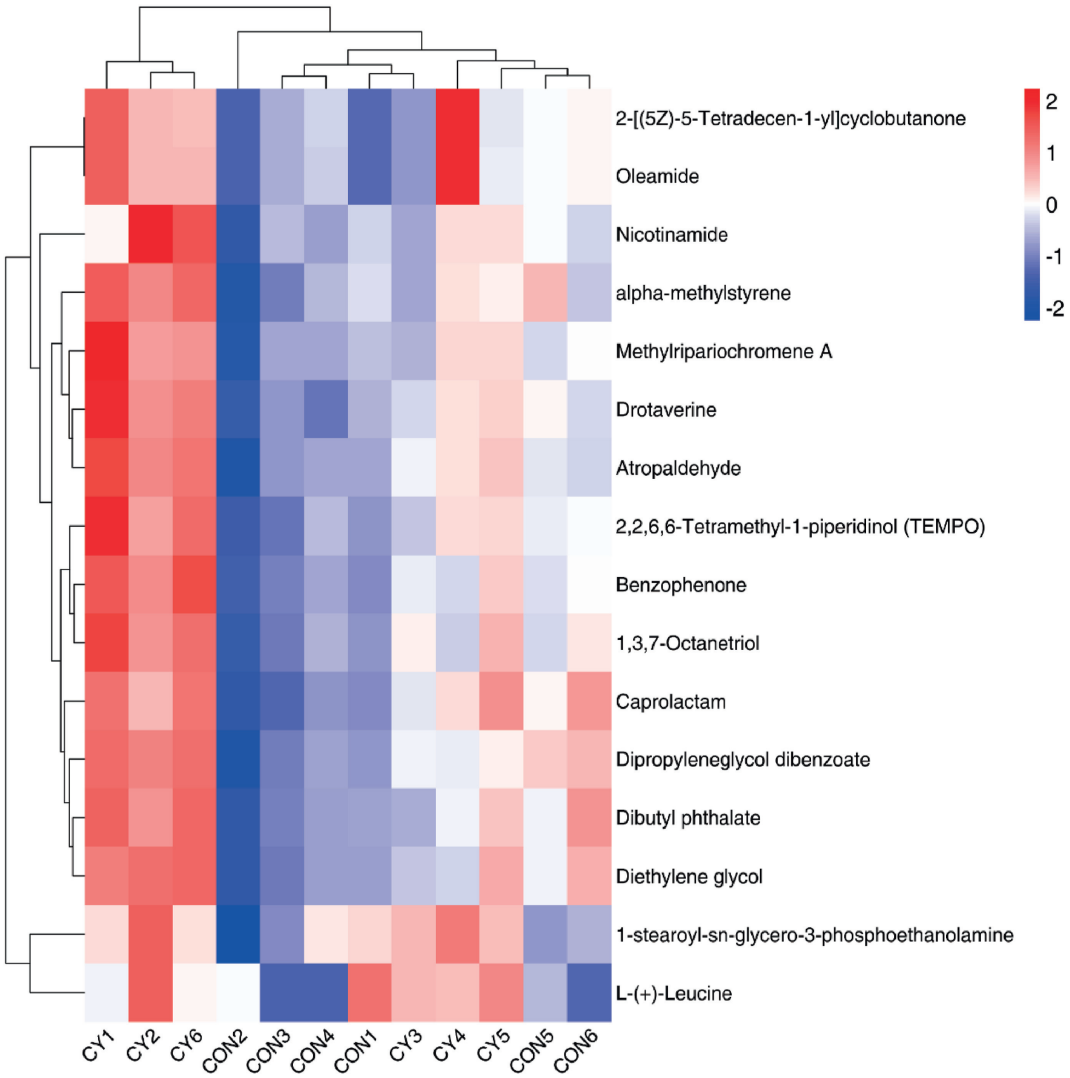

Fig. 3. Heatmap of the hierarchical clustering analysis for metabolites that were present at significantly different concentrations in the CON and CY groups ($P < 0.05$, VIP >1.0). CON: control group ($n = 6$, without chromium yeast supplementation); CY: chromium yeast group ($n = 6$, received additional chromium yeast at 0.36 mg Cr/kg DM).

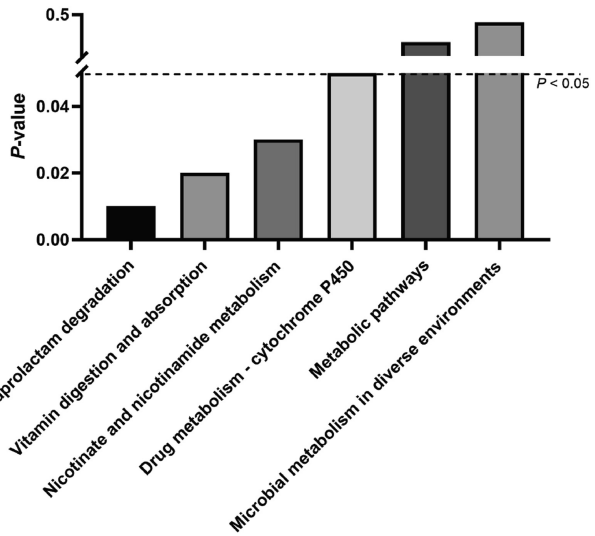

Fig. 4. Metabolic pathway analysis of the significantly different plasma metabolites between the two groups of dairy cows.

caprolactam degradation ($P = 0.012$), vitamin digestion and absorption ($P = 0.021$), nicotinate and NAM metabolism ($P = 0.026$), and drug metabolism-cytochrome P450 ($P = 0.047$, Fig. 4), along with the associated metabolites (Table 4).

3.4. Plasma NAM concentration

The plasma concentrations of NAM in the dairy cows were quantified using LC-MS/MS to validate the results of the metabolomic analysis, and this confirmed that the plasma NAM concentration was significantly higher in cows that had consumed the CY supplement (Fig. 5).

4. Discussion

Gradual increases in ambient temperature have direct adverse effects on dairy cows (Baile and Forbes, 1974). The stress response system of dairy cows begins to react at temperatures of 25 to 26 °C, and performance decreases rapidly at 30 °C (Rhoads et al., 2013). The meteorological data indicated that the cows were under heat stress in the present study, because the mean temperature exceeded 25 °C during the study period. The THI has been commonly

Table 4
Significantly different metabolites and KEGG pathways involved between the CON and CY groups.[1]

Significantly different metabolite	Fold change	KEGG pathway	*P*-value
Epsilon-caprolactam	1.58	Caprolactam degradation	0.012
		Metabolic pathways	0.205
		Microbial metabolism in diverse environments	0.420
Nicotinamide	1.76	Nicotinate and nicotinamide metabolism	0.026
		Metabolic pathways	0.205
		Vitamin digestion and absorption	0.021
Atropaldehyde	1.94	Drug metabolism–cytochrome P450	0.047

[1] CON: control group ($n = 6$, without chromium yeast supplementation); CY: chromium yeast group ($n = 6$, received additional chromium yeast at 0.36 mg Cr/kg DM).

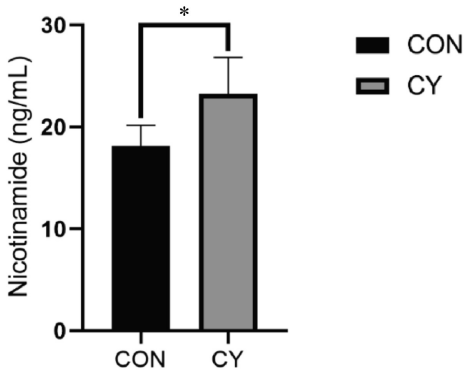

Fig. 5. Plasma concentrations of nicotinamide in each group of dairy cows. CON: control group ($n = 6$, without chromium yeast supplementation); CY: chromium yeast group ($n = 6$, received additional chromium yeast at 0.36 mg Cr/kg DM). *$P < 0.05$.

used as an index of the degree of heat stress in dairy cows (Collier et al., 1982a). Previous studies demonstrated that dairy cows can suffer from heat stress when the THI is greater than 68, and that they enter a mild state of heat stress when it exceeds 72 (Armstrong, 1994; Smith et al., 2013). This manifests in part as altered metabolism and reduced absorption (Cowley et al., 2015). In the present study, the THI exceeded 72 throughout the 8-week study period, and the RT and RR of the cows in the CON group were 39.6 °C and 79.3 breaths/min respectively, indicating that they were experiencing heat stress. The RT and RR are accepted means of evaluating heat stress in dairy cows, because RR increases to improve heat dissipation, made necessary by the high core temperature, indicated by a high RT (Brown-Brandl et al., 2005).

CY is obtained from yeast cells cultured in a Cr-rich medium, which has dual functions of yeast culture and Cr (Zhang et al., 2021). To minimize the effect of yeast on heat-stressed dairy cows, yeast culture was added in TMR. Therefore, all dairy cows in the 2 groups consumed similar amount of yeast (from TMR and CY), and the only difference was whether the dairy cows were supplemented with Cr or not. Previous studies have shown that supplementation with Cr reduced the RR of heat-stressed dairy calves (Kargar et al., 2018; Mousavi et al., 2019). Shan et al. (2020) demonstrated that supplementation with CY at 0.18, 0.36, or 0.54 mg Cr/kg DM reduces the RR and RT of dairy cows in both a linear and quadratic manner. Consistent with these findings, in the present study, supplementation with CY reduced the RT and tended to decrease the RR of the cows, which implies that CY helps in heat dissipation to alleviate the negative impacts of heat stress.

Previous studies of the effects of Cr supplementation on the lactation performance of dairy cows have yielded inconsistent results. McNamara and Valdez (2005) demonstrated that Cr propionate, providing 10 mg/d of Cr, did not affect milk yield. Leiva et al. (2017) also found no differences in milk yield or composition when dairy cows were fed 2.5 g/d of Cr propionate. By contrast, Nikkhah et al. (2011) showed that supplementation with Cr methionine increased milk yield and the protein and lactose percentages in the milk of heat-stressed lactating dairy cows, which is consistent with our findings that milk yield and the milk protein and lactose percentages were increased by dietary CY supplementation in heat-stressed dairy cows. These inconsistent findings might be explained by differences in the chemical form and dose of Cr, as suggested by Bin-Jumah et al. (2020). In the present study, the increases in milk yield and protein content induced by CY supplementation might be associated with the increased nitrogen utilization in vivo, because a lower urea nitrogen concentration was observed in the serum of dairy cows. Further study is still needed to investigate the underlying mechanism of CY on protein metabolism.

The lower lactose synthesis induced by heat stress is an important cause of the reduction in milk production (Wheelock et al., 2010; Baumgard and Rhoads, 2013), and might be explained by the lower serum glucose concentration of heat-stressed dairy cows, because blood glucose is the principal precursor for lactose synthesis, and lactose concentration is the principal determinant of the osmotic pressure and yield of milk (Baumgard and Rhoads, 2013; Zhang et al., 2014). Under heat stress, dairy cows are less able to mobilize lipids than at normal temperatures, such that they rely more on glucose for energy (Slimen et al., 2016). Trivalent Cr plays an important role in the regulation of blood glucose (Zhang et al., 2014), and the maintenance of an appropriate blood glucose concentration indirectly increases the milk lactose concentration of dairy cows, whether heat-stressed or not (Subiyatno et al., 1996; Kafilzadeh et al., 2012; Shan et al., 2020). Kargar et al. (2018) demonstrated that 0.05 mg/kg $BW^{0.75}$ Cr supplementation increases the serum glucose concentration of calves under heat stress, and Yari et al. (2010) also showed that Cr supplementation increases the serum glucose of preweaning calves. In the present study, supplementation with CY increased serum glucose concentration and milk lactose content because it provides precursors for the synthesis of milk lactose and increases glucose uptake by mammary gland cells.

As discussed above, the disturbances in glucose, lipid, and protein metabolism induced by heat stress might be a result of alterations in endocrine function that negatively affect the secretion of hormones (McDowell et al., 1976). Dietary Cr influences glucose metabolism because it increases cellular glucose uptake by promoting the binding of insulin to its extracellular receptors (Chen et al., 2006; Vincent, 2015; Bin-Jumah et al., 2020), which indirectly results in a lower insulin concentration in the circulation. The Cr should have increased the concentration of insulin and ratio of insulin to glucose, but decreased the concentration of glucose, showing a greater insulin sensitivity of dairy cows, as described by Hayirli et al. (2001). In the present study, no difference was observed in the ratios of insulin to glucose between the 2 groups, indicating that CY supplementation did not affect insulin sensitivity of heat-stressed dairy cows. However, the serum insulin

concentration of the cows was reduced by CY supplementation. Nikkhah et al. (2011) found that serum insulin concentration is reduced by the administration of 0.05 mg of Cr/kg $BW^{0.75}$ to early-lactation heat-stressed dairy cows. Kumar et al. (2015) also reported that supplementation with Cr reduces the circulating insulin concentration of heat-stressed calves. Kargar et al. (2018) reported that supplementation with Cr reduces the circulating insulin concentration of dairy cows.

In addition, thyroid hormones (T3 and T4) regulate metabolic activity, especially glucose and lipid metabolism (Gu et al., 2021). The thyroid gland principally secretes T4, whereas most T3 (approximately 80%) is produced by the deiodination of T4 (Sapin and Schlienger, 2003). Therefore, T4 represents a useful indicator of thyroid hormone production. A high endogenous T4 concentration or the administration of T4 have been shown to stimulate lactation (Blaxter et al., 1949). However, heat-stressed dairy cows tend to have low T3 concentrations and high T4 concentrations, in order to reduce heat production (Collier et al., 1982b), and in the present study, T3 concentration was reduced, whereas that of T4 was increased by CY administration.

To further investigate the metabolic mechanism whereby CY supplementation alleviates heat stress in dairy cows, we utilized LC-MS/MS to identify plasma metabolites. Sixteen metabolites that were present at significantly different concentrations in the 2 groups were identified, of which NAM, which is involved in the KEGG pathway of nicotinate and NAM metabolism, was found to be present at a high concentration in the supplemented cows and was selected for further investigation. NAM, also known as niacinamide, is the amide form of vitamin B_3, and NA is the alternative form of the water-soluble vitamin B_3 (Maiese, 2020). NAM is the precursor of nicotinamide adenine dinucleotide (NAD^+, coenzyme I) and is required for the synthesis of nicotinamide adenine dinucleotide phosphate ($NADP^+$, coenzyme II). NAD^+ and its derivatives serve as coenzymes for oxidoreductases and dehydrogenases, which are responsible for essential metabolic processes, such as glycolysis, the citric acid cycle, and mitochondrial electron transport (Cui et al., 1995). NAM can increase NAD^+ supply, provide the respiratory chain with extra NAD^+, improve glucose utilization, and prevent oxidative stress (Mitchell et al., 2018; Wei et al., 2018a; Maiese, 2020). To date, NAM has been used in dairy cows to improve performance, prevent negative energy balance, and regulate glucose and lipid metabolism (Wrinkle et al., 2012; Wei et al., 2018a, 2018b). Several previous studies have shown that NA or NAM increases glucose concentrations in thermoneutral or heat-stressed dairy cows (Di Costanzo et al., 1997; Jaster and Ward, 1990; Wei et al., 2018a). In the present study, supplementation with CY increased the plasma NAM concentration of dairy cows. NAM has been reported to mediate a transient, local vasodilatory effect (Ogawa et al., 2007), which contributes to heat dissipation (Hirst et al., 1995; Burns et al., 1997). The increased plasma NAM played a role as a potent vasodilator and helped the heat dissipation of the cows, thus contributing to the reduction in RT, resulting in subsequent improvement in serum glucose concentration and the lactation performance of heat-stressed dairy cows.

5. Conclusions

In summary, we have demonstrated that dietary supplementation with CY reduces RT and improves the performance of mid-lactation dairy cows during heat stress by increasing milk yield and the milk protein and lactose contents and yields. Supplementation with CY influences both the endocrine function and glucose metabolism of heat-stressed dairy cows: it increases the serum glucose and T4 concentrations, but reduces those of urea nitrogen, insulin, and T3. In addition, CY supplementation increases the plasma NAM concentration, which plays a role as the potent vasodilator and to dissipate heat from the body, thus contributing to the reduction in RT, the regulation of glucose homeostasis and an improvement in the lactation performance. These findings indicate that CY ameliorates the metabolic effects of heat stress and improves glucose metabolism, which helps to alleviate the negative effects of heat stress and improve the lactation performance of mid-lactation dairy cows.

Author contributions

Yeqianli Wo and **Fengtao Ma:** Data curation, Investigation, Methodology, Writing – original draft. **Qiang Shan:** Investigation, Methodology, Writing – review & editing. **Duo Gao** and **Yuhang Jin**: Investigation, Methodology. **Peng Sun:** Supervision, Writing – review & editing, Funding acquisition, Project administration, Conceptualization.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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Appendix supplementary data

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