

Effects of Dietary N-Carbamylglutamate Supplementation on Milk Production Performance, Nutrient Digestibility and Blood Metabolomics of Lactating Holstein Cows Under Heat Stress

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Abstract

Background: Heat stress (HS) becomes a serious threat to dairy cow industry in certain circumstances, N-carbamylglutamate (NCG) is a novel feed additive that may be used to alleviate HS. However, the effects and mechanisms of NCG on dairy cows under HS are still unknown. A total of 48 Holstein dairy cows with similar days in milk (154.37 ± 13.56 d), parity (1-3), and body condition score (BCS) were randomly divided into 4 groups of 12 animals each. Under HS, the cows were fed a TMR diet supplemented with 0 (control), 15, 20, 25 g of NCG/d per cow for 60 days. Milk production performance was recorded and serum parameters were examined. Meanwhile, metabolomics study of plasma based on liquid chromatography–mass spectrometry (LC-MS) was also applied to investigate metabolites and pathways in response to NCG supplementation.

Results: Milk yield (MY) was increased but milk urine nitrogen (MUN) was reduced by the NCG treatment. Addition of 15 g of NCG/d increased the milk protein percentage (MPP) compared with the control. No effect of NCG was observed on dry matter intake (DMI), lactose percentage (LP), milk fat percentage (MFP) and somatic cell count (SCC). Serum glucose (GLU) levels in cows fed with 15, 20, 25 g of NCG/d were increased by 14.35%, 19.34% and 18.63% in comparison with the control. Meanwhile, the concentrations of blood urea nitrogen (BUN) and blood ammonia (BA) were decreased with the addition of NCG. Results for antioxidant capacity and immune function showed beneficial effects of NCG, such as the increases in total antioxidant capacity (T-AOC), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), IgG, IgM and IgA. Clear separations of plasma metabolic profiles between control and NCG groups were observed in the score plots. Sixteen different metabolites involved in metabolisms of amino acids, ketone bodies, butanoate and energy, as well as gut microbiome-derived metabolism were regulated by NCG supplementation.

Conclusions: This study found that NCG treatment improved antioxidant capacity, immune function, production performance, and metabolic profile of dairy cows under HS and provided new evidence for the better utilization of NCG.

Background

Arginine participates in protein synthesis and metabolism to improve intestinal absorption function, hormones secretion and nitric oxide (NO) production, which ultimately benefits the growth, development and production performance of dairy cows [1, 2]. However, with the high arginase activity or rumen degradation, arginine is rapidly degraded with short biological half-time. Furthermore, the high dietary level of arginine may cause an absorption disorder of lysine due to the competition for the common transporters [3]. Moreover, arginine is uneconomically used in cows for its high cost. Due to these disadvantages, promoting the endogenous arginine synthesis will be an economic and alternative strategy to increase the supply of arginine for the animals.

The limiting factor in urea cycle of arginine synthesis is the availability of N-acetylglutamate (NAG) which is unstable and easily hydrolyzed to acylamino acids [4]. N-carbamylglutamate (NCG) is an analogue of NAG, which can enter the mitochondria to activate carbamylphosphate synthase-1 (CPS-1) and therefore promote the synthesis of arginine. In addition, NCG is stable in the ruminal environment and does not antagonize other amino acids such as lysine and histidine [5]. Thus, oral supplementation of NCG can increase the endogenous synthesis of arginine. At present, NCG as a promising feed additive, is widely used in farm animals production. Previous studies have found that dietary supplementation of NCG could promote the metabolism of urea cycle and the uptake and utilization of nitrogen in sheep [6]. Moreover, several results also suggested that NCG have positive effects on growth promotion, tissue development and reproductive performance in piglets, sheep, goats and broilers [7].

As global warming intensifying, heat stress (HS) in farm animals including the dairy cows becomes more problematic. When the temperature-humidity index (THI) value is greater than 72, dairy cows will have a decline in dry matter intake (DMI) or even refuse to eat, which leads to a decreased milk yield (MY) [8]. Moreover, a series of metabolic disorders, such as mental malaise, anorexia, shortness of breath, acceleration of pulse times, and rise of body temperature, could also occur in the dairy cows under HS. All of these may lead to detriments to the immunity and redox equilibrium [9]. Additionally, recent work has shown that milk composition and reproductive capacity of dairy cows are affected by HS besides the MY reduction [10].

Metabonomics studies the entire metabolic map and functional regulation in organism through the qualitative and quantitative analyses of the metabolites [11]. With high sensitivity and high resolving power, liquid chromatography-mass spectrometry (LC-MS)-based metabolomics is now widely applied as a versatile tool to screen the changes in diverse biological systems [12, 13]. Through mining the metabolic pathways such as sugar generation, metabolism of amino acids and lipids, physiological status under diseases, stress and nutrients in dairy cows can be systematically evaluated by metabonomic method [14, 15]. In dairy cows, main

biofluid samples used in metabonomic studies are rumen fluid, blood and milk. Blood metabolites are easily affected and can be directly used to characterize metabolic pathways related with dietary treatment [16].

Although the physiological function of NCG has been demonstrated, its effects on dairy cows under HS remain unclear. This study attempted to increase the understanding of the physiological processes associated with NCG treatment and provide theoretical guidance for the application of NCG in dairy cows. The present study investigated the effects of dietary supplementation of NCG in dairy cows during lactation under HS. Milk yield and composition, blood biochemistry, HS indicators, antioxidant and immune indexes were evaluated in the experiment. With the application of LC-MS based metabonomics, the plasma metabolites associated with NCG treatment were identified.

Methods

Animals, diets and experiment design

NCG was produced by Beijing Animore Sci. & Tech. Co., Ltd. (Beijing, China). Forty-eight Holstein dairy cows with similar days in milk (154.37 ± 13.56 d), parity (1–3), and body condition score (BCS) were provided by Hong Da dairy farm (Baoding, Hebei). The cows were randomly separated into 4 groups ($n = 12$ per group) as: control (TMR diet), low dose NCG (LNCG, TMR with NCG 15 g/day per cow), medium dose NCG (MNCG, TMR with NCG 20 g/day per cow) and high dose NCG (HNCG, TMR with NCG 25 g/day per cow). Experimental design was shown in Fig. 1. The detail composition and nutrient level of the basal diet were given in Table S1. The NCG treatment lasted for 60 days. THI was employed as an index of the HS status of the dairy cows. In this study, the THI of cowsheds ranged from 72 to 87.7 (Figure S1), indicating the dairy cows were clearly exposed to HS over the course of the experiment.

Sample Collection And Measurements

Samples of the TMR diets and orts were collected three times every week throughout the experiment period. Feed samples were dried (65°C , 48 h) and ground for the analyses of DMI, crude protein (CP), ether extract (EE), neutral detergent fiber (NDF), acid detergent fiber (ADF) [17], and contents of calcium (Ca) and phosphorus (P). The measurement methods were accorded to the AOAC [18]. In addition, NDF and ADF were measured by the using of ANKOM-A2000i fiber analyzer (Ankom Technology, Macedon, NY).

Milk yield of each cow was recorded electronically three times every day. Milk samples were collected at the time points of 15, 30, 45 and 60 d through milking at 0600, 1400, and 2000 h in the experiment, and the samples were collected into the aliquot of 50 mL at the proportion of 4:3:3. Milk compositions including fat percentage (MFP), protein percentage (MPP), lactose percentage (LP), somatic cell count (SCC) and urea nitrogen (MUN) were analyzed at the DHI Central of Hebei Animal Husbandry Varieties Workstation (Shijiazhuang, China).

Blood samples (10 mL) were collected from the coccygeal vein into vacuum tubes with or without heparin before morning feeding at the middle (30 day) and end of the experiment (60 day), and then centrifuged (2000 g , 4°C and 15 min) to obtain the serum and plasma, respectively. The plasma and serum samples were stored at -80°C until analysis. Serum was used to measure blood biochemistry indexes including glucose (GLU), urea nitrogen (BUN), total protein (TP), albumin (ALB), creatinine (CRE), aspartate aminotransferase (AST), alanine aminotransferase (ALT) and ammonia (BA) by the using of biochemistry analyzer Microlab-300. In addition, antioxidant and immune indexes and heat stress indicators were measured by the corresponding colorimetric methods or ELISA kits provided by Nanjing Jiancheng Biology Engineering Institute (Nanjing, China). Heat stress indicators were composed of triiodothyronine (T_3), thyroxine (T_4), growth hormone (GH), prolactin (PRL), heat shock protein-70 (HSP-70), cortisol and ascorbic acid. Antioxidant indexes were consisted of total antioxidant capacity (T-AOC), glutathione peroxidase (GSH-Px), malondialdehyde (MDA) and superoxide dismutase (SOD). Immunoglobulins (Ig) including IgG, IgM and IgA were also examined.

Sample Preparation For Metabonomics

Plasma samples were thawed at room temperature prior to analysis. 200 μL of plasma was placed into PE tubes, and then 400 μL of methanol/water (4:1, v/v) was added. Next, the tubes were vortex-mixed and incubated for 1 h at -20°C , and then centrifuged at $14,000\text{ g}$ for 20 min at 4°C . The supernatant was collected and dried. Lyophilized powder samples were reconstituted by dissolving

in 100 μ L of 60% methanol. The samples were vortexed (1 min) and centrifuged (14,000 g , 15 min, 4 $^{\circ}$ C) to collect the supernatant for metabolomic analysis. Quality control (QC) samples prepared from the pooled supernatant were placed in the analysis sequence.

Metabonomic Data Analysis

Chromatographic separation of plasma was performed with Vanquish ultra high performance liquid chromatograph (UHPLC) system (Thermo, USA). An Accucore HILIC column (2.1 \times 150 mm, 2.6 μ m) was used in the study. The column was maintained at 40 $^{\circ}$ C, and the flow rate was 0.3 mL/min. The mobile phase for the positive ion mode was consisted of A (0.1% formic acid, 95% acetonitrile, 10 mM ammonium acetate) and B (0.1% formic acid, 50% acetonitrile, 10 mM ammonium acetate); the negative ion mode was consisted of A (95% acetonitrile, 10 mM ammonium acetate) and B (50% acetonitrile, 10 mM ammonium acetate). The process of linear gradient elution was shown as follows: 0–1 min, 98% A; 1–17 min, 98 – 50% A; 17–17.5 min, 50% A; 17.5–18 min, 50–98% A; 18–20 min, 98% A.

Mass spectrometer used in this study was Q Exactive™ HF-X (Thermo, USA) equipped with an electrospray ionization source (ESI) operating in positive (ESI+) and negative ion modes (ESI-). The MS main properties were set as follows: spray voltage, 3.2 KV; sheath gas flow rate, 35 arb; aux gas flow rate, 10 arb; scan range, m/z of 100–1500; capillary temperature, 320 $^{\circ}$ C. MS/MS data was acquired in data dependent acquisition (DDA) mode.

Metabonomic data processing and metabolites identification

The MS data was processed by Compound Discovery 2.0 (Thermo, USA) for nonlinear alignment, automatic integration and extraction of the peak intensities. Then, the obtained dataset was imported into SIMCA-P (Umetrics AB, Sweden) for multivariate statistical analysis. Principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) were performed. Cumulative R^2 and Q^2 of the PCA and OPLS-DA models were used to evaluate the fitness and predictive capability of the model. Variable importance in projection (VIP) score in the OPLS-DA model was constructed, and the P -value of the candidate metabolite was analyzed by Student's t -test. Metabolite with $VIP > 1$ and $P < 0.05$ was considered to be statistically significant.

According to the exact molecular weight and MS/MS fragmentation pattern data, metabolite identification was carried out through the database search including HMDB (<http://www.hmdb.ca/>), METILN (<https://metlin.scripps.edu>), mzCloud (<https://www.mzcloud.org/>), and Chemspider (<http://www.chemspider.com/>). KEGG (<https://www.kegg.jp/>) and HMDB search was conducted to identify the metabolic pathways and biochemical reactions of the metabolites. Pathway analysis was performed by MetaboAnalyst 4.0 (<http://www.metaboanalyst.ca/>) for further biological interpretation.

Statistical analysis

All the data were analyzed using the MIXED procedure in the SAS software version 9.4 (SAS Institute Inc., Cary, NC). In MIXED model, NCG supplementations were fixed effects and cows were random effects. The PDIFF option adjusted by the Tukey method was included in the LSMEANS statement to account for multiple comparisons among treatments. The linear and quadratic responses to increasing NCG supplementation doses were determined by using specific preplanned contrasts. Data were presented as mean and mean standard error (SEM). Treatment effects were declared significant at $P \leq 0.05$, and trends were discussed at $0.05 < P \leq 0.10$.

Results

Milk production performance and nutrients apparent digestibility

NCG treatment significantly increased MY and reduced MUN ($P < 0.05$) (Table 1). Compared with the control, MPP in LNCG increased by 7.36%. Meanwhile, low, medium, and high doses of NCG reduced MUN by 17.73%–13.46%–16.31%, respectively, as compared with the control. No significant difference was observed among DMI, LP, SCC, and MFP among NCG-treated groups and control. Quadratic response to the increasing NCG was observed for MY ($P = 0.04$), MPP ($P = 0.01$), and MUN ($P = 0.09$). Results of the MY suggested that NCG at the dosage of 20 g/day per cow was optimal. In addition, NCG had no effects on the nutrients apparent digestibility ($P > 0.05$) (Table S2).

Table 1
Effects of different NCG addition on DMI and performance of lactating Holstein cows.

Items	Groups				SEM	Probability		
	Control	LNCG	MNCG	HNCG		P-NCG	Linear	Quadratic
DMI, kg/d	22.67	23.42	23.21	22.70	1.16	0.96	0.89	0.59
MY, kg/d	29.90 ^b	31.32 ^a	31.48 ^a	31.20 ^a	0.278	∞0.01	∞0.01	0.04
MFP, %	3.64	3.73	3.70	3.67	0.081	0.90	0.69	0.52
MPP, %	3.27 ^b	3.49 ^a	3.36 ^{ab}	3.35 ^b	0.046	0.03	0.18	0.01
LP, %	5.00 ^{ab}	5.02 ^a	4.94 ^b	4.95 ^{ab}	0.026	0.10	0.10	0.26
SCC, 10 ⁴ /ml	4.65 ^a	3.89 ^{ab}	3.33 ^b	3.65 ^{ab}	0.381	0.09	0.02	0.54
MUN, mg/dL	13.37 ^a	11.00 ^b	11.57 ^b	11.19 ^b	0.431	∞0.01	∞0.01	0.09

DMI: dry matter intake, MY: milk yield, LP: lactose percentage MFP: milk fat percentage, MPP: milk protein percentage, MUN: milk urine nitrogen, SCC: somatic cell count. In the same row, different small letter superscripts mean $P < 0.05$.

Blood Biochemistry

The levels of GLU in LNCG, MNCG and HNCG were significantly higher than those in the control with increasing by 14.35%, 19.34% and 18.63% ($P < 0.05$) (Table S3). The BUN and BA were markedly reduced in NCG treated groups ($P < 0.01$). Meanwhile, the levels of GLU and BA were linearly elevated and decreased, respectively ($P < 0.01$), in response to increase of NCG dosage. There were no significant differences in the TP, ALB, CRE, ALT, and AST among groups.

Antioxidant And Immune Indexes

As shown in Table 2, in comparison with the control group, the T-AOC, SOD, and GSH-Px levels were significantly increased ($P < 0.05$), and the MDA was markedly decreased in three NCG treatment groups ($P < 0.05$). T-AOC, SOD, and GSH-Px increased linearly ($P < 0.01$) and MDA decreased linearly ($P < 0.01$) with increasing NCG dosages. The levels of T-AOC, SOD, and GSH-Px in HNCG group were significant higher and MDA was lower than those in LNCG or MNCG groups ($P < 0.05$), which showed a dose-effect relationship between antioxidant indexes and NCG. It was found that the values of IgA and IgG in all NCG groups were higher than those in the control ($P < 0.05$). The levels of IgM were not different among control, LNCG and HNCG, but it was markedly higher in MNCG ($P < 0.05$). IgA and IgM showed a quadratic response ($P < 0.01$) and IgG was linearly increased ($P < 0.01$) with increasing dosages of NCG. Moreover, the high values of IgG, IgA, and IgM in MNCG group suggest an optimal NCG dosage for immunity enhancement.

Table 2
Effect of NCG on antioxidant and immune indexes.

Items	Groups				SEM	Probability		
	Control	LNCG	MNCG	HNCG		P-NCG	Linear	Quadratic
T-AOC, U/mL	34.14 ^c	43.74 ^b	45.49 ^b	48.41 ^a	0.960	∞0.01	∞0.01	0.47
SOD, ng/mL	6.66 ^c	8.77 ^b	9.64 ^a	10.18 ^a	0.242	∞0.01	∞0.01	0.87
GSH-Px, ng/mL	3,277 ^c	3,621 ^b	4,343 ^a	4,501 ^a	83.87	∞0.01	∞0.01	∞0.01
MDA, mmol/mL	8.25 ^a	7.26 ^b	6.75 ^b	4.55 ^c	0.184	∞0.01	∞0.01	∞0.01
IgA, mg/mL	13.33 ^d	15.53 ^c	25.06 ^a	23.15 ^b	0.477	∞0.01	∞0.01	∞0.01
IgM, mg/mL	11.80 ^b	12.23 ^b	15.34 ^a	11.89 ^b	0.360	∞0.01	0.01	∞0.01
IgG, mg/mL	23.30 ^d	26.49 ^c	30.28 ^a	28.00 ^{bc}	0.658	∞0.01	∞0.01	0.12

T-AOC: total antioxidant capacity, SOD: superoxide dismutase, GSH-Px: glutathione peroxidase, MDA: malondialdehyde. In the same row, different small letter superscripts mean $P < 0.05$.

Hs Indicators

As shown in Table 3, the values of T₃, GH, PRL, and ascorbic acid were significantly higher but cortisol was markedly lower in all three NCG groups than that in the control ($P < 0.05$). There were no differences in HSP-70 and T₄ between control and the NCG treatment groups. Linear responses to increasing NCG dosages were obtained for T₃, PRL, HSP-70, cortisol, and ascorbic acid ($P < 0.01$). Meanwhile, NCG had quadratic significant effect on GH ($P < 0.01$). Notably, the levels of HSP-70, ascorbic acid, and T₃ in MNCG and HNCG were higher and the cortisol was lower than those in the LNCG ($P < 0.05$).

Table 3
Effect of NCG on heat stress indicators.

Items	Groups				SEM	Probability		
	Control	LNCG	MNCG	HNCG		P-NCG	Linear	Quadratic
Cortisol, ng/mL	119.18 ^a	96.00 ^b	68.97 ^c	74.73 ^c	2.25	∞0.01	∞0.01	0.63
T ₃ , nmol/L	2.77 ^d	4.30 ^c	4.90 ^b	5.63 ^a	0.124	∞0.01	∞0.01	0.19
T ₄ , nmol/L	160.36	157.87	151.99	156.32	5.19	0.71	0.40	0.81
GH, ng/mL	7.61 ^c	10.52 ^b	11.84 ^a	10.51 ^b	0.288	∞0.01	∞0.01	∞0.01
PRL, mIU/L	342.70 ^c	453.70 ^b	461.47 ^b	487.62 ^a	8.55	∞0.01	∞0.01	0.05
HSP-70, pg/mL	252.19 ^{ab}	247.58 ^b	280.81 ^a	268.63 ^a	6.25	∞0.01	0.01	0.32
Ascorbic acid, ng/mL	14.58 ^c	16.33 ^b	19.23 ^a	19.79 ^a	0.385	∞0.01	∞0.01	0.03

T₃: triiodothyronine, T₄: thyroxine, GH: growth hormone, PRL: prolactin, SOD: superoxide dismutase, HSP-70: heat shock protein-70. In the same row, different small letter superscripts mean $P < 0.05$.

Metabolites Identification Associated With Ncg Treatment

In order to identify the metabolites related with the NCG treatment, the PLS-DA models were established. According to the PLS-DA score plots in ESI+ and ESI- modes, a clear separation between control and NCG-treated groups was observed (Figure S3). Either in

PCA or PLS-DA score plots, it was found that the separation trend between control and NCG-treated groups was good, which indicated that the NCG could cause the level changes of some potential metabolites. Based on the VIP > 1 and $P < 0.05$, the different metabolites were selected. Sixteen different plasma metabolites associated with NCG were listed in Table 4. It was found that the concentrations of 8 metabolites significantly increased and 8 metabolites remarkably decreased in NCG-treated groups compared with the control (Table 4). Based on the metabolite abundance, the hierarchical clustering analysis was carried, and the identified metabolites were visualized in a clustering heatmap (Fig. 2A). In the heat map, the dendrogram showed that the samples in the control group were clustered, and the samples in NCG-treated groups were separated from the control. Meanwhile, the metabolites with similar abundance patterns were also closely positioned.

Table 4
Significantly modulated metabolites in cows related with NCG based on PLS-DA.

No.	Formula	MW	RT	Metabolite	Fold Change			KEGG Pathway
					LNCG/C	MNCG/C	HNCG/C	
1	C ₆ H ₁₄ N ₂ O ₂	146.10529	0.91	Lysine	0.90	0.51*	0.56	Lysine metabolism
2	C ₅ H ₁₀ O ₃	118.0628	1.713	3-Hydroxyvaleric acid	0.82	0.65*	0.84	-
3	C ₂₂ H ₄₄ NO ₇ P	465.2842	13.555	LysoPC (14:1)	1.36	1.80**	1.00	Glycerophospholipid metabolism
4	C ₂₀ H ₃₄ O ₂	306.255	14.518	Linolenic acid	0.88	1.09	0.61*	Linoleic acid metabolism
5	C ₁₂ H ₂₂ O ₄	230.15093	11.976	Dodecanedioic acid	1.29**	1.62*	1.49**	-
6	C ₂₆ H ₄₃ NO ₆	465.3075	12.868	Glycocholic acid	2.08*	2.03**	2.03*	Primary bile acid biosynthesis
7	C ₄ H ₅ N ₃ O	111.04307	1.087	Cytosine	1.30	0.89	1.56**	Pyrimidine metabolism
8	C ₉ H ₈ O ₃	164.04707	1.391	2-Hydroxycinnamic acid	0.74	0.51**	0.86	Phenylalanine metabolism
9	C ₉ H ₈ O ₂	148.0528	0.38	Cinnamic acid	1.29	1.90*	2.88	-
10	C ₁₆ H ₃₀ O ₂	254.2245	0.211	Palmitelaidic acid	0.60	0.29*	0.48	-
11	C ₆ H ₁₁ NO ₂	129.0792	1.59	Pipecolic acid	0.85	0.65**	1.03	Lysine degradation
12	C ₂₆ H ₄₃ NO ₅	449.3148	12.866	Glycoursodeoxycholic acid	1.81*	1.81**	1.66	-
13	C ₄ H ₇ N ₃ O	113.0592	0.088	Creatinine	1.49*	1.56**	2.49	Arginine and proline metabolism
14	C ₂₄ H ₄₀ O ₆	424.2832	11.813	Hydroxycholic acid	1.99*	2.49	1.78*	-
15	C ₁₁ H ₁₂ N ₂ O ₂	204.0903	5.36	Tryptophan	0.59*	0.84	0.84	Amino acid metabolism
16	C ₄ H ₈ O ₃	104.0476	1.097	3-Hydroxybutyric acid	0.87	0.66*	0.87	Butanoate metabolism

RT: retention time; MW: molecular weight; -: not available in the KEGG pathway; Compared with the control, * $P < 0.05$, ** $P < 0.01$.

Pathway Analysis

The metabolites were checked against the KEGG database to find out the corresponding metabolic pathways and the biological pathways related with the metabolites were listed in Table 4. The pathway analysis was also performed by MetaboAnalyst to identify and visualize the metabolic pathway associated with NCG treatment. The summary of the pathway analysis results was shown in

Table S4. Figure 2B showed that the pathways in response to NCG treatment were lysine degradation, synthesis and degradation of ketone bodies, aminoacyl-tRNA biosynthesis, metabolisms of linolenic acid, butanoate, glycerophospholipid, and tryptophan.

Discussion

HS in dairy cows causes reductions in milk yield and quality and reproductive performance, which lead to a tremendous economic loss to dairy cow producers. Moreover, the metabolomic disturbances induced by HS also make the dairy cows more susceptible to diseases [19]. Severe energy reduction caused by reduced DMI is responsible for the above changes of the dairy cows under HS [20]. However, some studies suggested that HS-induced decrease in DMI accounted for only approximately 35–50% of the MY reduction [21]. In present study, the DMI in NCG-treated groups were tended higher than in the control, but not significant. The results of markedly increased MY in NCG groups under HS are consistent with previous findings that lactation performance of dairy cows could be improved by the NCG supplementation at 20 g /day per cow [22]. It suggests that the increased MY in NCG groups may be not closely related with DMI, but through the improvement of factors in blood biochemistry, oxidative stress, and serum metabolomic profile.

Arginine has been found to increase the milk protein yield and protein content in dairy cows. Supplementation of NCG can increase the concentration of arginine in blood and promote the synthesis of milk protein. In this study, the increase in MPP in NCG treatment groups was agreed with previous reports [23]. It was found that the MPP values in LNCG group were significantly increased compared with the control. This suggests that 15 g NCG/day to the cows is sufficient to enhance the endogenous synthesis of arginine. The decreases in levels of MUN, BUN and BA in blood in NCG groups indicate that NCG might improve the milk protein through the utilization of urea synthesis. Previous studies reported that HS affected the energy metabolism through the reduction of GLU concentration [24]. The increase of GLU in NCG groups in this study may be beneficial for the enhancement of energy metabolism and milk synthesis. Gu et al found that NCG addition could increase the milk fat content in mid-lactating dairy cows [23]. However, the MFP of the dairy cows under HS showed no changes between control and NCG groups in this study. The reason for this inconsistency might be attributed to the different physiological status and lactation stages.

HS can cause damage to immunity and induce oxidative stress in dairy cows [25]. Compromised immune function was also observed that the serum IgG concentration in calves under HS was significantly reduced [26]. Oxidative stress not only induces DNA damage and mitochondrial disorder, but also affects the protein synthesis and gene expression. Antioxidants including T-AOC, GSH-Px and SOD have been known to neutralize the detrimental effects of oxidative stress [27]. According to the results, the protective effects of NCG were detected where NCG supplementation increased the concentrations of T-AOC, GSH-Px and SOD and reduced the MDA in plasma of the cows under HS. These findings are consistent with those in the previous studies [28, 29]. Levels of immunoglobulins in serum are important indicators of immune response. Zhang et al reported that NCG could improve immune function in the intrauterine growth retarded lambs by the elevated plasma IgA concentration [30]. Consistent results were observed in present study that the levels of IgA, IgM, and IgG were elevated by NCG treatment indicating the improvement of immunity of dairy cows. The above findings demonstrate that NCG supplementation attenuated HS in dairy cows by increasing the production of antioxidants and the enhancement of immune response.

Various hormones, such as T_3 , T_4 and cortisol released from the endocrine system, are important to maintain the balance of body heat production and lose. Reduction of T_3 , increase of cortisol and the overexpression of HSP-70 are used as indicators for the animal exposure to HS [31]. Compared with the control, NCG significantly reduced the levels of cortisol and increased T_3 in this study. This indicates the counteracting of negative effects from HS. GH and PRL are involved in the energy metabolism in response to HS. Therefore, these hormones may be used to enhance the milk production. HS suppresses the production of GH and PRL and caused the alteration of energy metabolism [32]. Higher plasma concentrations of GH and PRL in NCG-treated cows than those in the control may explain the enhanced energy metabolism for milk synthesis and improved the productive performance of dairy cows under HS.

Endogenous metabolite alterations are associated with milk production reduction induced by HS [33]. In the present study, LC-MS based metabolomics was performed, and the metabolic profiling of plasma was analyzed to screen the metabolites related with NCG treatment. Sixteen different metabolites were identified and the pathway analysis indicated that these metabolites were potentially involved in metabolisms of amino acids, ketone bodies, butanoate, energy as well as gut microbiome-derived metabolism (Fig. 8). These findings help us to understand the underlying physiological mechanisms of NCG on dairy cows under HS.

NCG affected amino acid metabolism of dairy cows under HS. Tian *et al* found that HS could increase concentrations of amino acids in plasma such as lysine, arginine and isoleucine through the regulation of nitrogen metabolism or energy metabolism [34]. In present study, the changes of amino acids induced by HS were reversed by the NCG treatment where the levels of lysine, tryptophan and pipercolic acid were reduced. Consistent with our partial results, a previous finding revealed that NCG significantly reduced plasma lysine concentration in rats suffered with oxidative stress [35]. In present study, reduced levels of lysine, tryptophan and pipercolic acid in plasma were consistent with the function of NCG in increasing the MPP in cows. Milk protein synthesis can decrease the level of amino acids in plasma due to that the amino acids are converted into proteins. It was found that NCG could increase protein synthesis and nitrogen utilization in animals [22]. Therefore, NCG might increase the MPP through sufficient using of amino acids in dairy cows under HS.

Creatinine as a degradation product of creatine phosphate plays an important role in maintaining energy homeostasis. Increased concentration of creatinine in NCG treatment groups suggested that creatine phosphate mobilization was increased to satisfy the energy requirement in response to HS [36]. 3-hydroxybutyric acid is a ketone body, and its level can be increased with the promotion of fatty acid β -oxidation. The decreased level of 3-hydroxybutyric acid in the NCG treatment groups might be caused through the improvement of energy metabolism. Under the HS, decreased level of GLU implied that the energy production from glucose aerobic oxidation was inhibited, and the β -oxidation of fatty acids might be promoted to increase the energy production [37]. NCG treatment showed favorable inhibition of 3-hydroxybutyric acid indicating the depression of fatty acid β -oxidation. This might be related to the improvement of energy metabolism. Dodecanedioic acid is a dicarboxylic acid. It is rapidly oxidized in the peroxisomes and then degraded in the mitochondria by β -oxidation [38]. Marked increase of dodecanedioic acid in NCG groups also indicated the reduction of fatty acid β -oxidation. Notably, results of 3-hydroxybutyric acid agreed with the increase of dodecanedioic acid. According to these results, it was speculated that NCG could maintain energy homeostasis of the dairy cows in response to HS by meliorating the changes of dodecanedioic acid, creatinine and 3-hydroxybutyric acid.

As physiological detergent, bile acids are essential for the excretion, absorption, and transport of fats in the liver and intestine. This study found that NCG increased the concentrations of glycocholic, glyoursodeoxycholic and hydroxycholic acids. The increase of these bile acids in the NCG group could facilitate the digestion and intestinal absorption of hydrophobic nutrients, which might make positive effects for dairy cows against HS. Different species of bacteria such as *Bacteroides*, *Clostridium* and *Lactobacillus* are involved in bile acid metabolism, and thus the gut microflora can modify bile acid composition [39]. Alterations of these bile acids might also be associated with the interventional effects of NCG on gut microbiome-derived metabolism, which should be investigated in future studies. It has been found that cinnamic acid and its derivatives have anti-oxidative activities [40]. NCG improved cinnamic acid level in plasma compared with the control. This agreed with the improvement of antioxidant indexes and was beneficial for improving the ability of antioxidants from NCG to decrease the oxidative stress injury. However, the level of 2-hydroxycinnamic acid was reduced in NCG groups. The possible reason for this result was that chemical structure instability of 2-hydroxycinnamic acid which was an extremely weak basic compound, and NCG might aggravate its consumption to produce antioxidative effects.

Conclusions

The present study suggested that supplementation with NCG in dairy cows could effectively alleviate HS, enhance antioxidant capacity, and immune function. These were evidenced by the improvement of HS indicators, antioxidant and immune indexes. These changes were contributed to improving the production performance of the dairy cows under HS. In addition, sixteen metabolites were identified by the LC-MS metabonomic analysis indicating that NCG altered some metabolic processes, such as metabolisms of energy, amino acids and gut microbiome-derived metabolism. Metabolic pathway analysis provided new insights into the biological function of NCG in dairy cows under HS. This study provided new evidence for the better utilization of NCG in dairy cow industry.

Abbreviations

HS: heat stress; NCG: N-carbamylglutamate; BCS: body condition score; LC-MS: liquid chromatography–mass spectrometry; MY: milk yield; MUN: milk urine nitrogen; MPP: milk protein percentage; DMI: dry matter intake; LP: lactose percentage; MFP: milk fat percentage; SCC: somatic cell count; GLU: glucose; MUN: milk urea nitrogen; BUN: blood urea nitrogen; T-AOC: total antioxidant capacity; SOD: superoxide dismutase; GSH-Px: glutathione peroxidase; NO: nitric oxide; THI: temperature-humidity index; CP: crude protein; EE: ether extract; NDF: neutral detergent fiber; ADF: acid detergent fiber; BUN: blood urea nitrogen; TP: total protein; ALB:

albumin; CRE: creatinine; AST: aspartate aminotransferase; ALT: alanine aminotransferase; BA: blood ammonia; T₃: triiodothyronine; T₄: thyroxine; GH: growth hormone; PRL: prolactin; HSP-70: heat shock protein-70; MDA: malondialdehyde; ESI: electrospray ionization source; PCA: principal component analysis; PLS-DA: partial least squares discriminant analysis; VIP: variable importance in projection.

Declarations

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Author contributions

YXG and JGL designed the experiments. YL, LYR, and RTX conducted the experiment. YFC and NM prepared materials. LQH, RTX, NM and YL prepared the manuscript. YXG and JGL reviewed and improved the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The protocol for animal study was approved by Institutional Animal Care and Use Committee of Hebei Agricultural University and adhered to the guidelines of the Care and Use of Laboratory Animals of China.

Consent to publish

Not applicable

Competing interests

The authors declare that they have no competing interests

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Figures

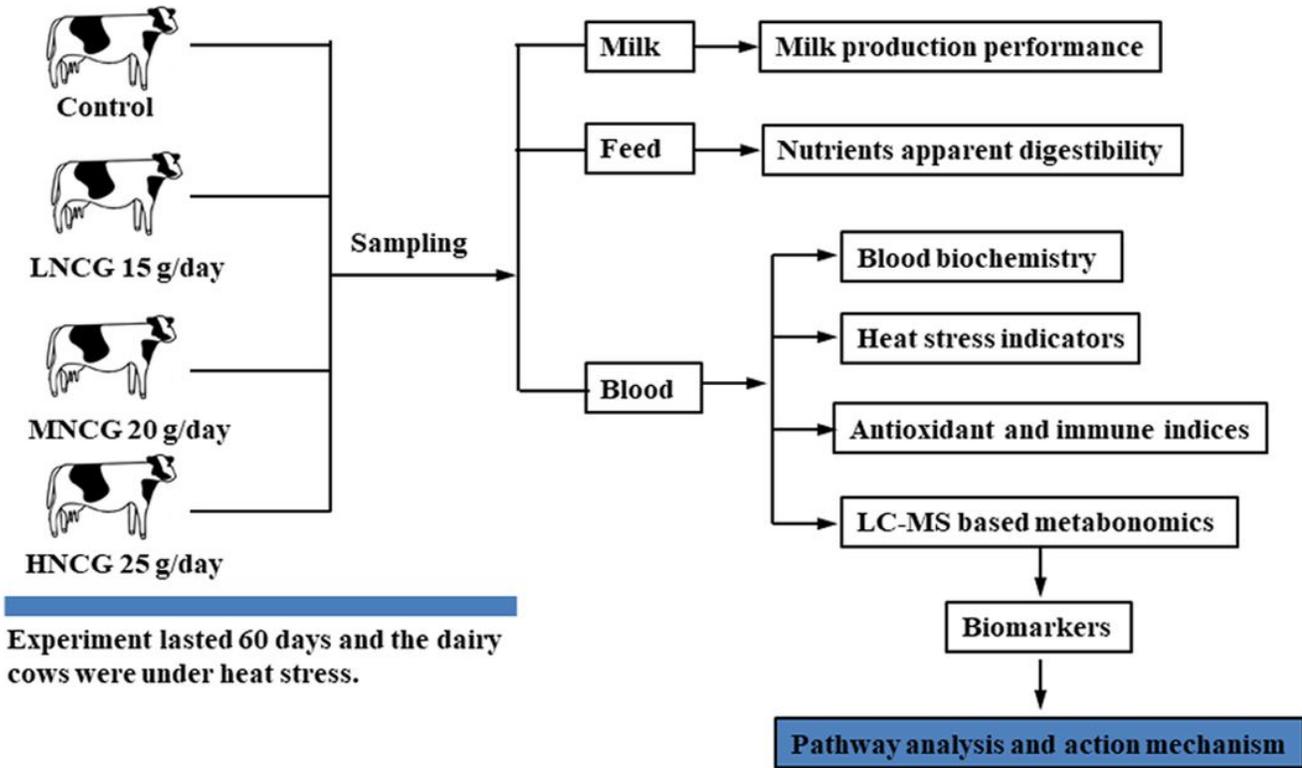


Figure 1

Flowchart of the experiment procedure.

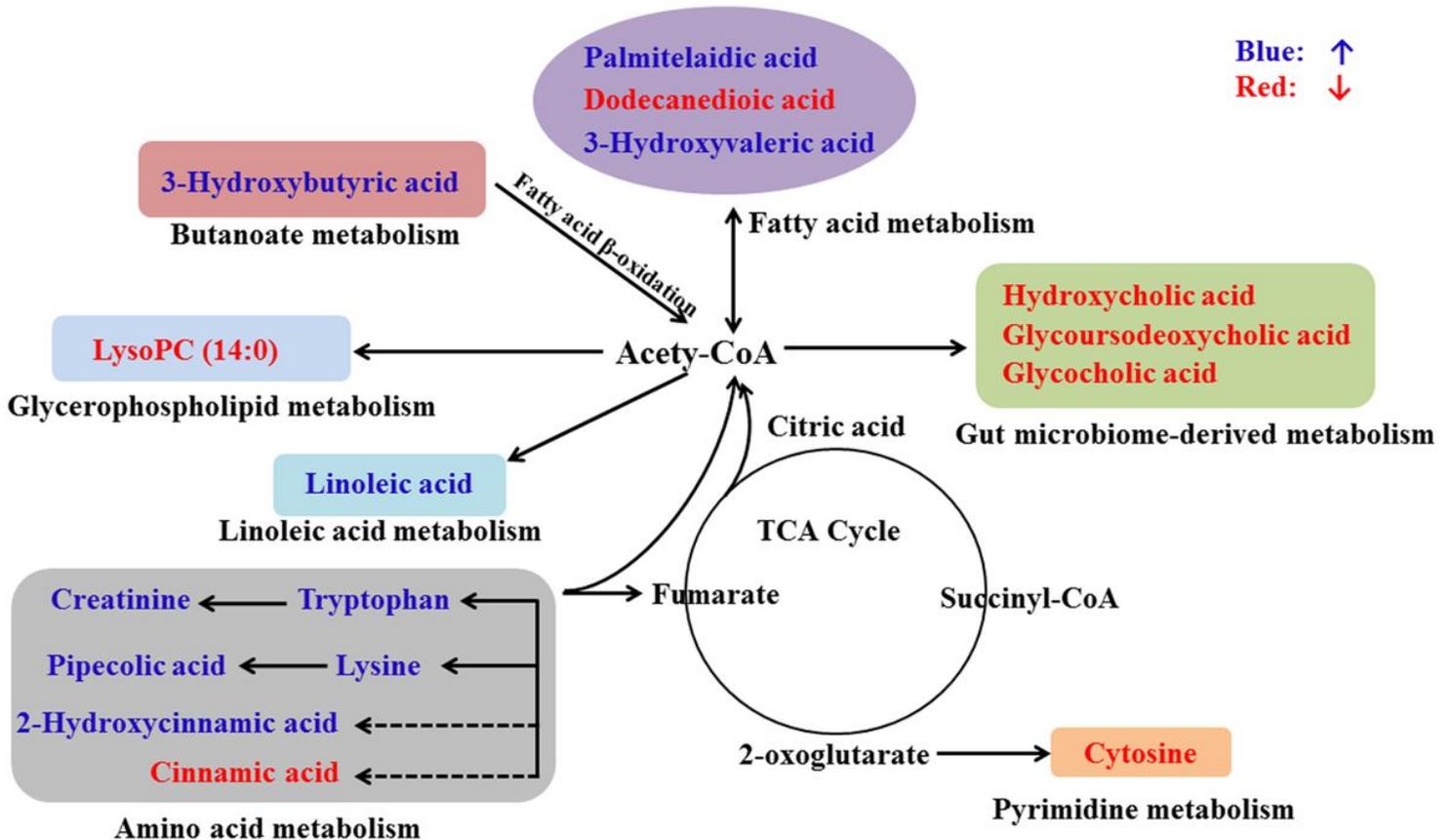


Figure 2

Clustering heatmap of the different metabolites and the metabolomics pathway analysis. A: Pearson's correlations and clustering heatmap of the different plasma metabolites. Metabolites were represented in rows and the samples from different group were represented in columns. Increasing intensities of the metabolites were indicated with blue to red colors. B: Pathway analysis performed by MetaboAnalyst Software based on the KEGG pathway library of Bos Taurus.

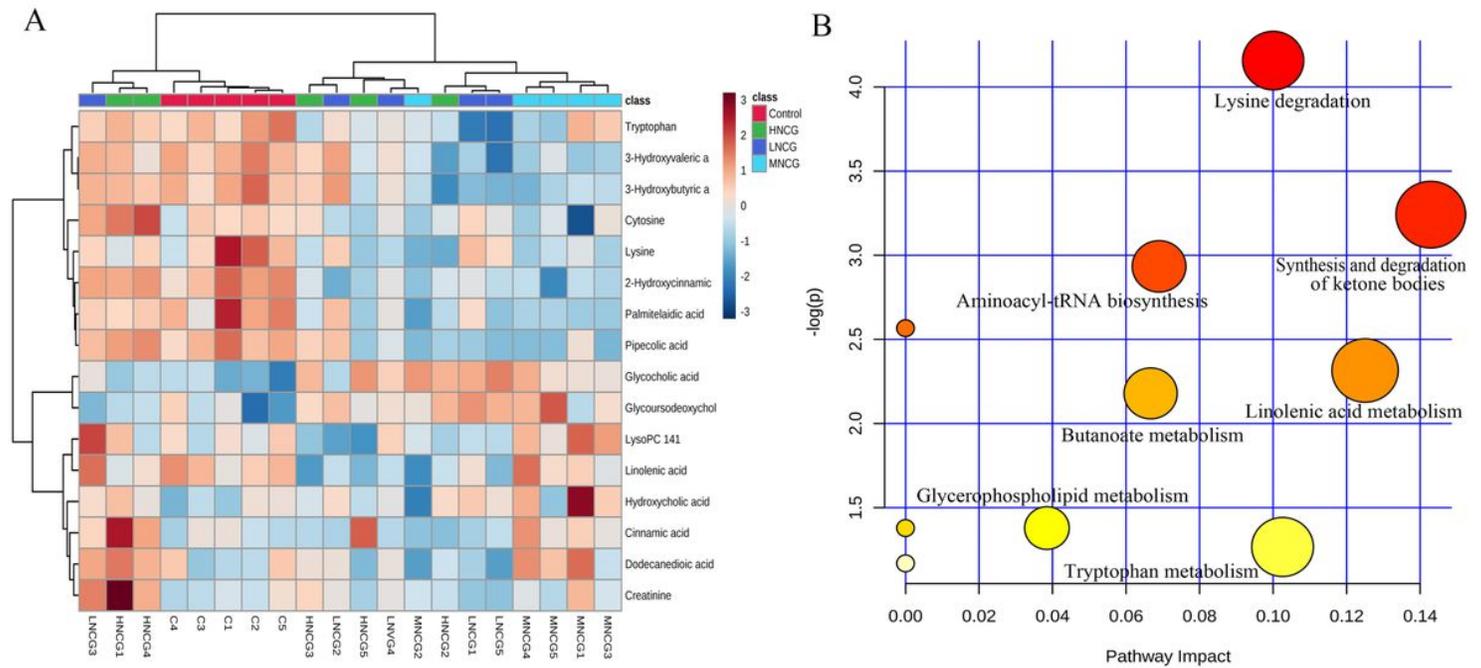


Figure 3

Integrative plasma metabolic pathway changes on the dairy cows under HS with NCG treatment. Potential metabolic pathways related with NCG in dairy cows under HS were established. Compared with the control group, change trends of the metabolites in NCG treatment groups were presented by different color that the blue and red indicated the increase and reduction, respectively.

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