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Original Research Article

Alterations in nutrient digestibility and performance of heat-stressed dairy cows by dietary L-theanine supplementation

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ABSTRACT

The purpose of this study was to investigate the effects of dietary L-theanine supplementation on apparent nutrient digestibility, milk yield, milk composition, and blood biochemical indices of dairy cows under heat stress. Thirty Chinese Holstein cows (19.84 ± 2.42 kg milk/d, 192.36 ± 40.77 d in milk and 2 ± 0.93 parities) were divided into 3 groups of 10 animals each. The control group was fed a basal total mixed ration (TMR) diet, while treatment 1 (LTA16) and treatment 2 (LTA32) groups were fed a basal TMR diet supplemented with L-theanine at 16 and 32 g/cow per day, respectively. The results showed that feeding the dairy cows with LTA16 treatment decreased ($P < 0.05$) their rectal temperature, whereas feeding with LTA32 treatment decreased ($P < 0.05$) their rumen fluid ammonia nitrogen content. In comparison to the control group, the supplementation of L-theanine had no significant effect ($P > 0.05$) on the dry matter intake, nutrient digestibility, total volatile fatty acid (TVFA) concentration and molar proportion of volatile fatty acid, milk yield, milk composition, feed efficiency and antioxidant capacity of the dairy cows. The triglyceride (TG) content of the LTA32 group was significantly greater ($P = 0.014$) than that of the control group. With the increase in L-theanine dosage, the serum cholesterol (CHOL) content significantly increased ($P = 0.013$). The serum albumin (ALB; $P = 0.067$), low-density lipoprotein cholesterol (LDL-C; $P = 0.053$), and high-density lipoprotein cholesterol (HDL-C; $P = 0.067$) contents showed an upward trend as L-theanine dosage increased. Ultimately, the results of this study show that supplementing dairy cow diet with L-theanine could decrease dairy cow rectal temperature, affect lipid metabolism, and potentially relieve the heat stress of dairy cows to some extent.

1. Introduction

Heat stress, which is a non-specific response of dairy cows to hot and humid environments, adversely affects the performance and physiological function of these animals (Yu et al., 2020). Further, it has also been reported that lactating cows are sensitive to heat stress, given that it is related to their physiological characteristics as well as their special nutritional requirements (Gantner et al., 2017). In general, metabolic thermogenesis typically arises in dairy cows during feed digestion and milk production. Heat stress occurs in dairy cows when their internally produced heat and absorbed ambient environmental heat exceeds that which they can be expelled through respiration, sweating, and breathing, particularly

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under high temperature and humidity conditions (Bernabucci et al., 2010). The effects of heat stress on lactating cows have been extensively studied due to the economic losses that they caused and their detrimental effects on the health of dairy cattle. Holstein cows are resistant to low temperatures, with an optimum survival temperature ranging from 5 to 25 °C. Cows suffering from heat stress during the summer exhibit increased respiration rates (Strong et al., 2015), decreased feed intake (Kim et al., 2021) and milk yields as well as changes in milk composition (Dikmen et al., 2020). Heat stress not only affects physiological indices and reduces production performance (Almuhanna et al., 2021), but also has effects on rumen metabolism (Zhao et al., 2019) and increases oxidative stress in dairy cows (Yu et al., 2020). Moreover, as global warming intensifies, cows are increasingly vulnerable to heat stress. Therefore, there is an urgent need for the identification of a kind of feed additive that can enable dairy cows resist heat stress.

L-Theanine (γ-glutamylethylamide), a unique non-protein amino acid found in tea, has lately received sizable attention due to its various biological activities. L-Theanine has anti-oxidation and anti-stress properties, and is able to enhance animal immunity. Previous studies have revealed that L-theanine dietary supplementation (200 mg/kg diet) can improve the growth performance of broilers (Saeed et al., 2018). In addition, theanine dietary supplementation (600 to 900 mg/kg diet) improved the antioxidant capacity and immunity of ducks (Zhang et al., 2019c). Moreover, dietary L-theanine was found to alleviate lipopolysaccharide-induced immunological stress in yellow-feathered broilers (Li et al., 2018a), and L-theanine could regulate glucose (GLU), lipid, and protein metabolism via insulin and AMP-activated protein kinase (AMPK) and their downstream signaling pathways in rat (Lin et al., 2020). Our previous study found that L-theanine supplementation at 16 g/d per cow reduced the lipopolysaccharide translocation into the peripheral blood and lipopolysaccharide accumulation in the milk, as well as mitigated lipopolysaccharide-induced inflammatory reactions in dairy cows during heat stress. However, the application of L-theanine has mainly focused on monogastric animals, but its effects on the nutrition utilization, physiology, and milk quality of dairy cows under heat stress is unclear. We hypothesize that L-theanine may relieve heat stress and improve milk quality by improving nutrient utilization and physiological conditions. Moreover, we aimed to provide scientific data and theoretical reference for L-theanine to relieve heat stress in dairy cows and the efficient utilization of L-theanine in ruminants.

2. Materials and methods

2.1. Animal ethics

All the experimental procedures carried out in this study were reviewed and approved by the Animal Care Committee of the College of Animal Science and Technology, Hunan Agricultural University, Changsha, China (20190718001).

2.2. Animals, experimental design, and diets

The study was conducted in the Deren Husbandry Dairy Farm (Changde, Hunan Province, China) during summer (from August to September 2019). The average temperature of the cowshed during the experimental period was 28.7 °C, the average humidity was 66.7%, and the average temperature and humidity index (THI) was 78.9. L-Theanine was obtained from the College of Horticulture and Landscape Architecture, Hunan Agricultural University (Hunan, Changsha, China), and the concentration of pure L-theanine was 98%.

Thirty lactating Chinese Holstein dairy cows with average values of 19.84 ± 2.42 kg milk/d, 192.36 ± 40.77 d in milk, and 2 ± 0.93 parities were used as experimental animals. The cows were randomly assigned into 3 groups (10 cows/group), each group fed with 1 of the 3 diets: basal total mixed ration (TMR) (control) or basal TMR supplemented with 16 g L-theanine/cow per d (LTA16) or 32 g L-theanine/cow per d (LTA32). L-Theanine was first mixed with a small amount of TMR and provided to each cow in a small basin for consumption and the remaining TMR was offered thereafter. At the same time, feed intake of dairy cows was recorded on group basis. The trial lasted 38 d, including 7 d of the pre-feeding period and 31 d of the formal trial period.

Cows were fed in stalls where they were given clean water to drink and were free to move about after being fed. The cowshed was equipped with an automatic excrement scraping floor, the juxtaposed hanging fans were started for 24 h, and the spraying facilities were regularly sprayed with water (50 s each time). During the experiment, the feeding management of each group of cows was consistent with the environment, and regular disinfection was carried out to keep the cowshed dry and hygienic. Cows were fed a TMR diets (05:00 and 17:30) and milked prior to feeding twice daily, and milk production was recorded daily. The ingredients and chemical compositions of the diets are presented in Table 1.

2.3. Temperature and humidity index measurements

Ambient temperature (Td, °C) and relative humidity (RH, %) of the cowshed were recorded daily (07:00, 13:00, and 20:00) using a thermometer and hygrometer attached to one instrument panel (Zhejiang Yiwu Equipment Co. Ltd., Zhejiang, China). The effective

Table 1
Composition and nutrient content in the basal diet (DM basis).

Item	Content
Ingredients, g/kg	
Corn silage	387
Alfalfa silage	71.2
Leymus chinensis hay	10.2
Alfalfa hay	142.5
Oat hay	10.2
Whole cottonseed	20.4
Beet pulp	16.3
Corn meal	134.4
Soybean meal	69.2
Brewer's spent grain	81.5
DDGS	14.3
Wheat bran	20.4
$NaHCO_3$	5.5
$Ca(HCO_3)_2$	5.1
NaCl	1.0
MgO	1.0
Limestone powder	3.7
Premix[1]	6.1
Nutrient levels, g/kg	
NE_L[2], MJ/kg	6.91
OM	916.9
EE	54.7
CP	172.1
NDF	440.2
ADF	286.7
Ca	7.6
P	3.9

DDGS = distillers' dried grains with solubles.
[1] Every 1 kg of premix contained 400 mg of Zn; 200 mg of Fe; 3,600 mg of Mg; 100 mg of Cu; 4 mg of Co; 96 mg of Cr; 350 mg of Mu; 50 mg of Se; 500 mg of lysine; 500 mg of methionine; 2,500,000 IU of vitamin A; 100,000 IU of vitamin D_3; and 4,000 IU of vitamin E.
[2] Net energy for lactating cow (NE_L) was calculated based on the Cornell–Penn–Miner Dairy system (CPM-Dairy; Boston et al., 2000).

scales were: Td = −30 to 50 °C and RH = 20% to 100%. The THI was calculated using the following equation (Dikmen and Hansen 2009):

$$THI = (1.8 \times Td + 32) - [(0.55 - 0.0055 \times RH)(1.8 \times Td - 26)]$$

2.4. Rectal temperature and respiratory rate

During the experiment, a timer was used every day (07:00, 13:00, and 20:00) to manually record the cows' respiration times per min. The respiration rate of the animals was recorded by counting the number of flank movements in 1 min. Rectal temperature was measured using an electronic thermometer 3 times per day (08:00, 14:00, and 20:00) during the last week of the experiment.

2.5. Feed and fecal samples collection and analysis

During the experiment, the feed intake and orts of each group were accurately recorded daily. Samples of the offered TMR and orts of each group during feces collection were collected mixed daily stored at −20 °C until analysis.

Fresh fecal samples were collected daily from each cow via rectal fecal collection through the rectum during the last 8 d of the experiment, after which the fecal samples for each group were pooled based on the sampleing date, mixed, and subsampled (800 g). Finally, the pooled fecal samples (800 g each group) were mixed with 10% sulfuric acid and stored at −20 °C until further analysis.

Forage and fecal samples were dried at 65 °C in a forced-air oven (101-3 A B, Tianjin Taisite Instrument Co., Ltd., Tianjin, China) to a constant weight. Samples were first ground to pass through a 40-mesh screen and then analyzed for dry matter (DM; method 930.15), crude protein (CP; method 2001.11), ether extract (EE; method 920.39), crude ash (Ash; method 942.05), neutral detergent fiber (NDF; method 2002.04), and acid detergent fiber (ADF; method 973.18) according to the procedures of AOAC (2005). The contents of calcium (Ca) and phosphorus (P) were measured using previously described methods (Tang et al., 2019; Wang et al., 2020a, 2020b), and gross energy was measured using an oxygen bomb calorimeter (SDACM3100, Hunan Sande Technology Co., Ltd., Hunan, China). The apparent digestibility of the nutrients was determined using the acid−insoluble ash internal marker method. The apparent digestibility of each nutrient was calculated using the following equation:

$$\text{Apparent digestibility of nutrients}(\%) = [1 - (A \times D)/(B \times C)] \times 100$$

where A is the insoluble ash content of hydrochloric acid in the feed, B is the hydrochloric acid insoluble ash content in the feces, C is the kind of nutrient content in the feed, and D is the nutrient content in the feces (Cheng et al., 2014).

2.6. Rumen fluid samples collection and analysis

Rumen fluid samples were collected before feeding in the morning using the oral stomach tube method. Briefly, the first 150 mL of rumen fluid was discarded. Thereafter, the subsequent 150 mL sample obtained was strained through 4 layers of cheese cloth, and ammonia nitrogen (NH_3−N) as well as volatile fatty acid (VFA) contents were determined as previously described (Wang et al., 2016). On the one hand, to determine the NH_3−N concentration, a UV-2300 spectrophotometer (Shimadzu, Kyoto, Japan) was used to record light absorbance at 700 nm. On the other hand, VFA concentrations were determined using an gas chromatograph (GC7890A, Agilent, Palo Alto, CA, USA) equipped with a hydrogen flame detector and a capillary column (30 m × 0.25 mm × 0.25 μm) as previously described.

2.7. Milk samples collection and analysis

The cows were milked twice daily (05:00 and 17:30), with yields recorded at each milking. Milk samples were collected from all cows on d 27 and 38 of the experimental period and analyzed for milk protein, milk fat, lactose, fat-free dry matter, total dry matter, and milk urea nitrogen (MUN) using an infrared spectrophotometer (Mino-78110 automatic milk composition analyzer, FOSS Company, Denmark). Subsequently, 4% fat-corrected milk (FCM) and energy-corrected milk (ECM) yields were calculated using the following equations (Tyrrell and Reid, 1965):

$$4\%\ FCM = 0.4 \times Milk(kg/d) + 15 \times Fat(kg/d)$$

$$ECM = 0.327 \times Milk(kg/d) + 12.95 \times Fat(kg/d) + 7.20 \times Protein(kg/d)$$

2.8. Blood samples collection and analysis

Before and 2 h after morning feeding, 10 mL blood samples were collected via the coccygeal vein of the cows using the disposable vacuum blood collection tubes (non-anticoagulant tubes) (Shandong Aosaite Medical Devices Co., Ltd.) on d 27 and 38 of the experimental period. Then the blood samples were centrifuged at 4 °C (1,500 × g) for 15 min, and serum was harvested and stored at −80 °C until further analysis. Simultaneously, 10 mL blood samples were collected using an anti-coagulation (heparin sodium) tube and centrifuged at 4 °C (3,500 × g) for 15 min. The resulting plasma was then stored at −80 °C until further analysis.

Serum total protein (TP), albumin (ALB), uric acid (UA), triglyceride (TG), cholesterol (CHOL), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein-cholesterol (LDL-C), and GLU levels were determined using an automatic blood biochemical analyzer (Cobas c311, Roche, Switzerland), according to the manufacturer's instructions (Roche Biochemical Kit). Total plasma superoxide dismutase (TSOD), glutathione peroxidase (GSHPX), and malondialdehyde (MDA) contents as well as its total antioxidant capacity (TAOC) were measured using a kit obtained from the Nanjing Jiancheng Institute of Biological Engineering in strict compliance with the operating procedures.

2.9. Statistical analysis

All data were analyzed using the MIXED procedure in SAS (ver. 9.4; SAS Institute, Cary, NC, USA). For the dry matter intake, nutrient digestibility and feed efficiency, the fixed effects of sampling date were included in the model. For milk production, 4% FCM, and ECM data, the fixed effects of parity and diet were included in the model. When analyzing milk production, the statistical model also included the average milk production in the 5 d before the experiment as a covariant. For the milk composition and serum biochemical index, the fixed effects of parity, diet, sampling time, and the interactions between diet and sampling time were included in the statistical model, and sampling time was considered as the repeated measurement. Animals were considered as the random effects. The Tukey's test was used to compare the differences among the 3 treatments. Orthogonal polynomial contrasts were used to analyze the linear and quadratic effects of the L-theanine levels. The contrast coefficients of the orthogonal polynomial were corrected using the IML procedure of SAS. Least square means are reported throughout

the text, and statistical significance was declared at $P < 0.05$, while a tendency was declared at $0.05 \leq P \leq 0.10$.

3. Results

3.1. Environmental temperature and humidity index

During the experimental period, the THI varied from 73.26 to 80.36, 74.05 to 85.36, and 73.39 to 84.61 in the morning (07:00), noon (13:00), and afternoon (20:00), respectively (Fig. 1). The mean daily maximum and minimum THI were 83.81 and 73.98, respectively.

3.2. Rectal temperature and respiratory rate

As shown in Table 2, the mean values of rectal temperature in the control, LTA 16, and LTA32 groups were 38.91, 38.67, and 38.85 °C, respectively. The rectal temperature of the cows of the LTA16 groups was significantly lower ($P = 0.015$) than that of the cows of the control group. The mean respiratory rate values in the control, LTA16, and LTA32 groups were 68.29, 64.38, and 66.85 flank movements per min, respectively. However, statistical analysis showed that there was no significant difference ($P = 0.331$) among the 3 groups (Table 2).

3.3. Dry matter intake and diet nutrient digestibility

The dry matter intake and apparent total-tract digestibility of nutrients had no significant ($P > 0.05$) effect in either the LTA16 or LTA32 groups in compared to those in the control group (Table 3). Nevertheless, the NDF and ADF of the LTA16 group were 4.16% and 4.74% and those of the LTA32 groups were 3.1% and 3.09% higher ($P > 0.05$) than those of the control group, respectively (Table 3).

3.4. Rumen fermentation parameters

As shown in Table 4, the NH_3–N concentration corresponding to the LTA32 treatment was significantly lower ($P = 0.035$) than that corresponding to the control group. However, the 3 groups exhibited no significant differences with respect to TVFA content, molar proportions of acetate, propionate, isobutyrate, butyrate, and valerate, and the acetate to propionate (A:P) ratio. Further, as the L-theanine dosage increased, the molar proportion of acetate ($P = 0.048$) increased linearly, whereas isovalerate level ($P = 0.031$) decreased linearly (Table 4).

3.5. Milk yield, milk composition, and feed efficiency

Increasing L-theanine tended to linearly increase the milk yield ($P = 0.077$), 4% FCM ($P = 0.071$), and ECM ($P = 0.098$), and linearly increased milk protein ($P = 0.041$), solid non-fat ($P = 0.048$), and total solid contents ($P = 0.035$) (Table 5). The MUN content in the treatments of LTA16 and LTA32 groups were lower ($P = 0.015$) than that in the control group.

3.6. Blood biochemical indices and the antioxidant indices

Data corresponding to blood biochemical indices, as well as the antioxidant index, are shown in Table 6. The serum TG content of the LTA32 group was significantly higher ($P = 0.014$) than that of the control group. Furthermore, serum CHOL content significantly increased ($P = 0.013$) with increasing L-theanine. In addition, the serum ALB ($P = 0.067$), LDL-C ($P = 0.053$) and HDL-C ($P = 0.067$) contents showed an upward trend as L-theanine increased. Although the 3 groups exhibited no significant differences in GSHPX, TSOD, and MDA contents as well as TAOC, increasing L-theanine dosage linearly decreased GSHPX ($P = 0.044$) and MDA ($P = 0.046$) contents.

4. Discussion

4.1. Environmental temperature and humidity index

The THI is used in livestock breeding as one of the main environmental indicators to assess possible heat stress in cows. Typically, a THI ≥72, indicates cows suffering from heat stress (Rees et al., 2016). For example, Collier et al. (2012) found that high-yielding cows (>35 kg/d) showed heat stress responses at a THI ≥68. In this present study, the mean daily maximum and minimum THI was 83.81 and 73.98, respectively. Indicating that the cows were subjected to heat stress throughout the experimental period. However, we show that the supplementation of 16 g L-theanine/cow per d significantly reduced rectal temperature in dairy cows compared to those in the control group, suggesting that L-theanine

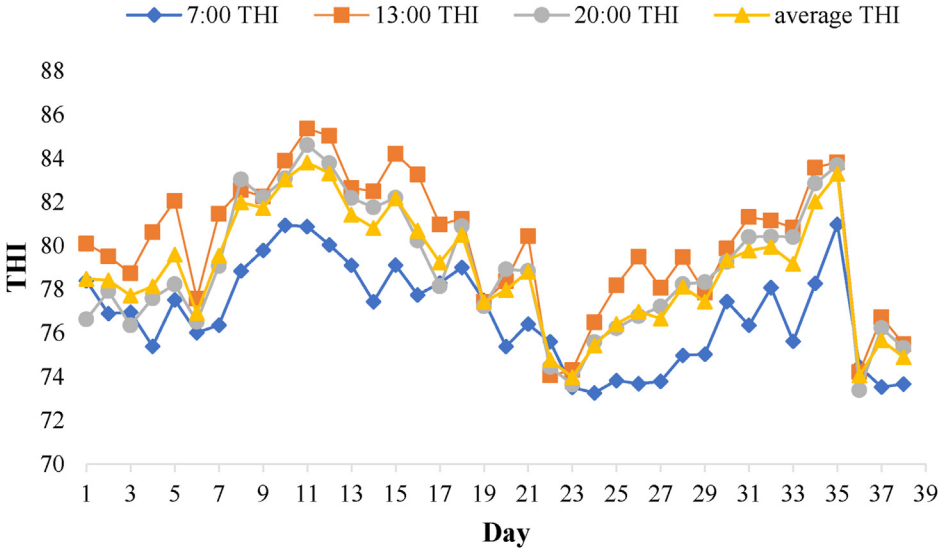

Fig. 1. Temperature and humidity index (THI) of the cowshed during the experiment.

Table 2
Effects of the different levels of L-theanine on the respiratory rate and rectal temperature of dairy cows during heat stress.

Item	Treatment[1]			SEM	*P*-value		
	Control	LTA16	LTA32		Treat	Linear	Quadratic
Respiratory rate, breaths, min	68.29	64.38	66.85	1.320	0.331	0.517	0.183
Rectal temperature, °C	38.91[a]	38.67[b]	38.85[a]	0.042	0.015	0.348	0.006

LTA = L-theanine.
[1] Cows were fed a basal diet (control) or a basal diet supplemented with 16 or 32 g of L-theanine/cow per day.

Table 3
Effects of different levels of L-theanine on the dry matter intake and nutrients apparent digestibility of dairy cows during heat stress.

Item	Treatment[1]			SEM	*P*-value		
	Control	LTA16	LTA32		Treat	Linear	Quadratic
Dry matter intake, kg/d	17.80	18.20	17.40	0.248	0.114	0.233	0.084
Dry matter, %	85.70	85.97	85.20	0.686	0.728	0.614	0.542
Gross energy, %	63.95	63.78	61.79	1.589	0.573	0.347	0.643
Organic matter, %	63.34	62.60	61.02	1.604	0.586	0.317	0.832
Crude protein, %	66.79	66.62	64.06	1.467	0.355	0.203	0.513
Neutral detergent fiber, %	64.25	68.41	68.99	3.031	0.495	0.282	0.634
Acid detergent fiber, %	63.24	66.34	66.33	2.577	0.626	0.407	0.628
Ether extract, %	81.82	79.65	78.02	1.450	0.203	0.078	0.878

LTA = L-theanine.
[1] Cows were fed a basal diet (control) or a basal diet supplemented with 16 or 32 g of L-theanine/cow per day.

Table 4
Effects of different levels of L-theanine on rumen fermentation of dairy cows during heat stress.

Item	Treatment [1]			SEM	*P*-value		
	Control	LTA16	LTA32		Treat	Linear	Quadratic
NH_3–N, mmol/L	9.77[a]	8.08[ab]	7.69[b]	0.685	0.035	0.020	0.356
TVFA, mmol/L	93.1	95.6	91.7	4.61	0.748	0.806	0.456
VFA profile, mol/100 mol							
Acetate	63.8	64.1	66.1	0.90	0.114	0.048	0.329
Propionate	21.4	21.1	19.0	1.07	0.181	0.081	0.373
Isobutyrate	0.863	0.827	0.845	0.0253	0.383	0.556	0.260
Butyrate	11.2	11.3	11.5	0.41	0.835	0.563	0.813
Isovalerate	1.52	1.44	1.38	0.050	0.080	0.031	0.848
Valerate	1.22	1.25	1.17	0.044	0.348	0.423	0.184
A:P	3.10	3.12	3.48	0.187	0.223	0.113	0.338

LTA = L-theanine; TVFA = total volatile fatty acid; A:P = acetate-to-propionate ratio.
[a, b] Different lowercase letters within rows indicate significant difference at $P < 0.05$.
[1] Cows were fed a basal diet (control) or a basal diet supplemented with 16 or 32 g of L-theanine/cow per day.

treatment could alleviate heat stress in dairy cows. We propose that the mechanism underlying this heat-stress alleviation may be related to L-theanine inhibiting the excitability of the sympathetic nervous system in heat-stressed dairy cows (Ozeki et al., 2008). This, in turn, results in blood vessel dilation and improved transfer of heat to the environment, ultimately alleviating the negative effects of heat stress in dairy cows. However, owing to the limited availability of information on the application of L-theanine for the management of heat stress in dairy cows, further investigation is necessary to verify this assumption.

4.2. Diet nutrient digestibility

Cows mainly feed on dry matter to meet their nutritional and energy requirements. However, heat stress decreases dry matter feed intake and nutrient digestibility in cows (Gao et al., 2017), resulting in insufficient nutrition and adverse effects on their

Table 5
Effects of different levels of L-theanine on the production variables of dairy cows during heat stress.

Item	Treatment[1]			SEM	*P*-value		
	Control	LTA16	LTA32		Treat	Linear	Quadratic
Milk yield, kg/d	18.60	19.00	19.60	0.440	0.212	0.077	0.773
FCR[2], kg/d	17.40	18.20	19.50	0.850	0.188	0.071	0.744
Energy-corrected milk[3], kg/d	19.60	20.40	21.70	0.940	0.247	0.098	0.734
Milk fat, %	3.79	4.07	4.24	0.229	0.297	0.121	0.783
Milk protein, %	3.48	3.58	3.75	0.104	0.093	0.041	0.702
Lactose, %	4.78	4.80	4.81	0.067	0.969	0.722	0.901
Solid nonfat, %	9.02	9.15	9.31	0.116	0.124	0.048	0.878
Total solid, %	12.60	12.90	13.30	0.267	0.102	0.035	0.882
Milk urea nitrogen, mg/dL	18.50[a]	17.40[b]	16.60[b]	0.538	0.015	0.004	0.863
Somatic cell count, lg/mL	2.12	2.18	1.96	0.127	0.210	0.274	0.239
Feed efficiency[4]	1.04	1.04	1.13	0.028	0.556	0.359	0.571

LTA = L-theanine.
[a,b] Different lowercase letters within rows indicate significant difference at $P < 0.05$.
[1] Cows were fed a basal diet (control) or a basal diet supplemented with 16 or 32 g of L-theanine/cow per day.
[2] FCR = 4% fat-corrected milk = 0.4 × Milk (kg/d) + 15 × Fat (kg/d).
[3] Energy-corrected milk = 0.327 × Milk (kg/d) + 12.95 × Fat (kg/d) + 7.20 × Protein (kg/d).
[4] Feed efficiency = Milk yield/Dry matter intake.

Table 6
Effects of different levels of L-theanine on blood biochemical indices of dairy cows during heat stress.

Item	Treatment[1]			SEM	*P*-value		
	Control	LTA16	LTA32		Treat	Linear	Quadratic
Total protein, g/L	71.35	73.60	71.43	0.992	0.193	0.952	0.070
Albumin, g/L	37.19	38.29	39.01	0.541	0.067	0.021	0.768
Blood urea nitrogen	4.16	4.11	4.21	0.140	0.870	0.822	0.635
Glucose, mmol/L	2.83	2.82	2.86	0.055	0.879	0.740	0.704
Uric acid, mmol/dL	0.81	0.77	0.79	0.025	0.410	0.577	0.225
Low-density lipoprotein cholesterol, mmol/L	1.40	1.65	1.64	0.080	0.053	0.042	0.178
High-density lipoprotein cholesterol, mmol/L	3.38	3.63	3.73	0.108	0.067	0.025	0.545
Triglyceride, mmol/L	0.14[b]	0.15[a,b]	0.16[a]	0.004	0.014	0.007	0.265
Cholesterol, mmol/L	3.82[b]	4.28[a]	4.35[a]	0.132	0.013	0.006	0.234
GSHPX, μmol/L	203	177	175	9.7	0.080	0.044	0.303
MDA, nmol/mL	4.43	4.02	3.52	0.312	0.133	0.046	0.900
TAOC, mmol/L	112	112	112	0.1	0.876	0.649	0.811
TSOD, U/mL	76.8	77.3	79.9	1.31	0.219	0.108	0.511

LTA = L-theanine; GSHPX = glutathione peroxidase; MDA = malondialdehyde; TAOC = total antioxidant capacity; TSOD = total plasma superoxide dismutase.
[a,b] Different lowercase letters within rows indicate significant difference at $P < 0.05$.
[1] Cows were fed a basal diet (control) or a basal diet supplemented with 16 or 32 g of L-theanine/cow per day.

overall body health. Studies suggest that L-theanine improves feed intake in broilers under lipopolysaccharide -induced immunological stress, and it can play a protective role in the growth and immune function of yellow-feathered broilers (Li et al., 2018b). L-Theanine also maintains the integrity of the intestinal mucosal barrier and promotes intestinal development (Wang et al., 2020). Moreover, L-theanine has been found to effectively improve jejunum morphology, as reflected by a decreased crypt depth and an increased villus height, villus height to crypt depth ratio, and goblet cell number in the jejunum (Zhang et al., 2019b). Thus, these factors may improve the digestion and absorption of nutrients.

Although a number of investigations have evaluated the nutritional properties of L-theanine used as livestock feed source, few studies have elucidated reported the digestion of L-theanine in ruminants. According to the findings of this study, an increase in L-theanine dose exhibited no significant effect on dry matter intake, apparent digestibility of gross energy, DM, Ash, CP, NDF, ADF, and EE. We hypothesize that this may be because of L-theanine degradation by rumen microorganisms. Therefore, we suggest the use of an L-theanine coating to prevent its degradation by rumen microorganisms.

4.3. Rumen fermentation parameters

The rumen of dairy cows is a stable ecosystem that provides a good metabolic environment for microbes, and maintaining its stability is of great significance (Carpinelli et al., 2021). Further, NH_3–N in the rumen of dairy cows, which is primarily generated by the degradation of ingested protein nitrogen and non-protein nitrogen, is the main nitrogen source for microbial protein synthesis (Nasrollahi et al., 2019). In this study, we observed that L-theanine supplementation (32 g/cow per day) under heat stress resulted in a decrease in rumen NH_3–N content. This decrease suggests that L-theanine possibly inhibits dietary protein degradability. Nasrollahi et al. (2019) reported that a decrease in rumen NH_3–N content with increasing diet fermentability indicates that the NH_3–N is used more efficiently for microbial protein synthesis. However, this could not be verified owing to insufficient literature; thus, further investigations are required.

Moreover, in the rumen, TVFA, which primarily contain acetate, propionate, and butyric acid, represent the final product of dietary carbohydrate fermentation (Shah et al., 2020). In this study, TVFA (acetate, propionate, isobutyrate, butyrate, valerate, and A:P) content remained unaltered with increasing L-theanine dose. However, isovalerate content showed a decreasing trend with increasing L-theanine dosage. Thus, L-theanine has no adverse effect on rumen fermentation in heat-stressed dairy cows.

4.4. Milk production

As previously alluded to, heat stress results in a decrease in the feed intake of dairy cows, and the ensuing insufficient nutrition results in cows not meeting their lactation requirements, ultimately leading to a decrease in milk production (Könyves et al., 2017; Mote et al., 2016; Nasr and El-Tarabany 2017). If cows are suffering from heat stress during the late gestation period, the regeneration of the mammary gland will be restricted, which is usually related to the dry period, and the milk yield will be reduced during the following lactation (Dahl et al., 2017). Previous studies have indicated that multiparous cows are more vulnerable to heat stress, and in some cases, multiparous cows were shown to lose more than 1 kg of milk per day (Bernabucci et al., 2014). This may be because multiparous cows typically produce more milk and thus generate more metabolic heat, ultimately adding to the likelihood of heat stress occurring. Ultimately, it is clear that heat stress has a dramatic effect on the milk yield of cows. In this present study, we found that dietary supplementation of L-theanine had no significant effect on the milk yield of dairy cows suffering from heat stress. This indicates that L-theanine was not able to improve the milk yield of heat-stressed cows. However, several previous studies have shown that L-theanine supplementation was able to improve growth performance and relieve heat stress in monogastric animals (Saeed et al., 2018). Zhang et al. (2020) found that L-theanine supplementation could increase the body weight of broilers at d 21 and 42. Moreover, Li et al., (2018b) found that dietary L-theanine alleviated the decreased body weight in lipopolysaccharide-induced immunological stress broilers. Furthermore, a recent study showed that L-theanine supplementation had a positive and cumulative effect on the growth performance of ducks, and that the appropriate level of L-theanine in the diet could significantly increase the body weight of ducks (Zhang et al., 2019b). The addition of dietary L-theanine is shown to improve growth performance, which may be because of its beneficial biological activities such as antioxidant activity and immunomodulatory function (Wang et al., 2020). Despite these previous findings, the results of this present study reveal that dietary L-theanine supplementation had no effect on the milk yield of heat-stressed dairy cows; as previously discussed we hypothesize that this may be due to L-theanine

degradation via rumen microorganisms. Whereas, further studies are needed.

4.5. Milk composition

Several investigators have reported that heat stress can alter the composition of milk by reducing milk protein, milk fat, lactose, solids-not-fat, and total solid contents (Gaafar et al., 2011) and increasing MUN content (Gao et al., 2016), leading to a decline in milk quality (Chanda et al., 2017; Gao et al., 2017). During the heat stress period, the mammary epithelial cells of dairy cows produce heat shock proteins to resist the effects of heat stress. The synthesis of heat shock proteins may decrease the utilization of amino acids for milk protein synthesis (Cowley et al., 2015), which will reduce the rate of milk protein (Hu et al., 2016). In Holstein cows from Luxembourg, Hammami et al. (2013) found that for every unit increase in THI, milk production decreased by 0.109 to 0.164 kg/d, milk fat decreased by 0.013 to 0.023 kg/d, and milk protein decreased by 0.01 to 0.013 kg/d. Interestingly, in this study, increasing the dose of dietary L-theanine resulted in upward trends in milk protein, solid non-fat, and total solids contents. Moreover, as the supplemental L-theanine dose increased, MUN was shown to decreased significantly. Overall, these results indicate that under heat stress conditions, L-theanine supplementation may improve milk composition to a certain extent, but the underlying mechanisms need further investigation.

4.6. Blood biochemical indices and the antioxidant indices

Heat stress can alter the physiological metabolic state of animals, which can be reflected by serum biochemical indices (Ihsanullah et al., 2017). Serum TP and UA levels are often used as indicators of amino acid utilization and protein decomposition (Donsbough et al., 2010). However, heat stress can cause a decrease in the total plasma protein levels in animals (Gudev et al., 2007). Zhang et al. (2019b) found that L-theanine administration could significantly reduce serum UA content and increase serum TP content of ducks. In this study, we found that the ALB content showed an upward trend with increasing L-theanine dose. This suggests that L-theanine can potentially increase protein synthesis and retention, which corroborates with the increase in milk protein content.

Fatty acids are obtained from TG, which are synthesized from acetyl-CoA by fatty acid synthase catalysis. TG are used to synthesize total cholesterol (TC) under the regulation of sterol element-binding protein 1c, sterolelement-binding protein 2, and low-density lipoprotein (LDL) receptor (Buga et al., 2008). L-Theanine has been shown to upregulate the expression of acetyl-CoA carboxylase 1 and fatty acid synthase at the mRNA and protein levels and promoted the formation of fatty acids (Xu et al., 2020). Furthermore, as the fatty acid level increased, the sterol element-binding protein 1c, sterolelement-binding protein 2, and LDL receptor mRNA and protein expressions is upregulated, leading to increased TC and TG contents (Xu et al., 2020). In contrast, some studies in mice, broilers, and rats reported that serum TG (Zheng et al., 2004), TC (Saeed et al., 2018), and high-density lipoprotein (HDL) (Yan et al., 2017) levels were significantly decreased upon L-theanine administration. Zhang et al. (2019b) found that supplementation with L-theanine (100 or 200 mg/kg diet) decreased TG, TC, and LDL-C contents in the serum of 28-d-old ducks; whereas it increased serum HDL and reduced LDL contents. Conversely, a higher level of L-theanine (300 mg/kg diet) exhibited the opposite effect, with lower HDL and higher LDL-C levels (Saeed et al., 2018). These contrasting results can be attributed to species-specific differences, growth stages at the time of assessment, and environmental conditions. Previous studies have also found that heat stress can affect lipid metabolism (Faylon et al., 2015) and reduce the plasma CHOL and TG contents in cows (Ronchi et al., 1999). In the present study, we found that different levels of L-theanine significantly increased the serum CHOL and TG contents in cows. Moreover, LDL-C and HDL-C levels showed an increasing trend. This indicates that dietary L-theanine supplementation can regulate the lipid metabolism in heat-stressed cows; however, the underlying mechanisms require further assessment.

At a suitable temperature, the oxidative system, as well as the antioxidant system, maintain a certain level of balance to prevent damage to body tissues. However, studies have shown that heat stress can induce hypoxia in body tissues, attenuate the activity of antioxidant enzymes, weaken the ability of cells to remove free radicals, thereby causing cause damage to the liver and other organs and tissues to varying degrees (Gursu et al., 2004). Further, heat stress results in an imbalance between the oxidative in the oxidation and antioxidant systems in cows, resulting in a decrease in serum T-AOC, SOD, and GSH-Px levels, and an increase in serum MDA content (Yu et al., 2020).

MDA is a product of lipid peroxidation, which is mediated by free radicals, and is often used as a marker of oxidative stress. Conversely, SOD and GSH-PX, which can protect animals from oxidative damage, are considered as key scavengers of reactive oxygen species (Inoue et al., 2013). Zhang et al. (2019a,b) reported that in the jejunum of ducks, dietary L-theanine significantly enhanced the activities of CAT, T-SOD, and GSH-PX, while significantly decreasing decreases MDA content. Similarly, in a study on mice treated with sodium dextran sulfate, Wang et al. (2016) observed that L-theanine supplementation can significantly inhibited MDA production, while enhancing the activities of T-SOD and GSH. Zhang et al. (2019a,b) also reported that supplementing the diet of broilers with 600 mg/kg L-theanine can reduce transport stress-induced damage on meat quality by improving antioxidant status in muscles. However, the results of this study indicated that dietary L-theanine had no significant effect on the plasma GSHPX, TAOC, TSOD and MDA contents of heat-stressed dairy cows. This may be because the antioxidative activities of L-theanine vary with animal species, test time, and the living environmental conditions of the experimental animals. However, further investigation is needed as the number of studies on the effects of L-theanine on antioxidant levels in heat-stressed dairy cows is limited.

5. Conclusions

Here, we show that dietary L-theanine supplementation decreased the rectal temperature, altered lipid metabolism and improved rumen fermentation in dairy cows suffering from heat stress. Interestingly, dietary L-theanine supplementation exhibited minor effects on the respiration rate, dry matter intake, apparent digestibility of nutrients, antioxidant capacity, milk yield, and milk composition of heat-stressed cows. We hypothesize that these minor effects are because of the degradation of L-theanine by rumen microorganisms. Nonetheless, further investigations are necessary to determine the mechanisms underlying these effects.

Author contributions

Lingmei Zhang and **Lingyuan Yang**: Data curation, Formal analysis, Investigation, Writing-original draft, Project administration. **Peihua Zhang** and **Yuli Zhou**: Project administration, Supervision. **Xingguo Huang**, **Qiongxian Yan** and **Zhiliang Tan**: Supervision, Funding acquisition. **Shaoxun Tang** and **Fachun Wan**: Conceptualization, Methodology, Formal analysis, Investigation,

Supervision, Project administration, Funding. All authors have read and agreed to the published version of the manuscript.

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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