Animal Nutrition 7 (2021) 1253–1257

Contents lists available at ScienceDirect

Animal Nutrition

journal homepage: http://www.keaipublishing.com/en/journals/aninu/

Short Communication

Alterations of endotoxin distribution across different biofluids and relevant inflammatory responses by supplementing L-theanine in dairy cows during heat stress

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ARTICLE INFO

Article history:
Received 25 July 2020
Received in revised form
22 February 2021
Accepted 10 March 2021
Available online 23 September 2021

Keywords:
Heat stress
Endotoxin
L-theanine
Inflammation

ABSTRACT

The present trial was performed to reveal the regulatory effects of L-theanine on the levels of lipopolysaccharide (LPS) endotoxin within different biofluids, as well as relevant inflammatory responses of dairy cattle under heat stress conditions. Thirty lactating Chinese Holstein dairy cattle (189 ± 47 days in milk, and 2 ± 1 parities) were allocated in a completely randomized design to each of 3 dietary treatments: the control (CON, 0 g/d per cow L-theanine), the low L-theanine dosage treatment (LL, 16 g/d per cow L-theanine), and the high L-theanine dosage treatment (HL, 32 g/d per cow L-theanine). This trial consisted of 38 d (7 d for adaption and 31 d for data and sample collection), and sample collection for rumen liquid, blood plasma or serum, and milk were conducted on the d 27 and 38, respectively. Dairy cattle were constantly exposed to environmental heat stress during this experiment according to the recorded temperature-humidity index (THI). In the LL treatment, LPS concentration in rumen liquid was higher ($P < 0.05$), whilst LPS densities in plasma and milk were lower ($P < 0.05$) than those of the CON. Supplementing L-theanine at 2 dosages both significantly lowered ($P < 0.05$) the level of interleukin (IL)-1β in the serum. Results of the present study suggested that L-theanine could be a promising additive in reducing the detrimental effects of heat stress on dairy cows, and L-theanine supplementation at 16 g/d per cow is preferred because it reduced the LPS translocation into the peripheral blood and LPS accumulation in the milk, as well as mitigated LPS-induced inflammatory reactions in dairy cows during heat stress. Further studies are necessitated to investigate the underlying mechanisms of L-theanine in LPS alteration and inflammation alleviation.

1. Introduction

It is widely acknowledged that dairy cows are vulnerable to heat stress, which could be characterized as an external condition that breaks the balance between heat load and heat emission causing hyperthermia of the animal (Fournel et al., 2017; Tao et al., 2018). Heat stress sets a major challenge and exerts vast economic impacts on the dairy industry in areas with high temperature and/or humidity (Leiva et al., 2017; Wheelock et al., 2010). Collier et al. (2017)

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Peer review under responsibility of Chinese Association of Animal Science and Veterinary Medicine.

Production and Hosting by Elsevier on behalf of KeAi

https://doi.org/10.1016/j.aninu.2021.03.012

previously reported that dairy cows under heat stress would normally experience losses in milk yield and reproductive performance, due to their elevated maintenance requirements. In addition, it has been reported that heat stress damages the barrier integrity of the intestinal tract and induces significant increment of the endotoxin (i.e., lipopolysaccharides [LPS]) flow across the mesenteric-drained and portal-drained viscera (Wang et al., 2011). Since the transfer of LPS to the interior circulation might stimulate the expression of pro-inflammatory cytokines and consequently result in systemic inflammation (Li et al., 2012), it could be inferred that heat stress can also cause bodily injury. Nutritional regulations could be adopted to complement the thermal environmental management in attenuating the harmful impacts of heat stress (Johnson, 2018). As the most plentiful amino acid in green tea leaves, L-theanine was found to benefit animal health through boosting immune function and mitigating stress (Alagawany et al., 2020; Li et al., 2016; Saeed et al., 2018; Tian et al., 2013), making it an alternative feed additive for heat stress-relieving in dairy cows. Nonetheless, little information is available about the influences of applying L-theanine in dairy cows under heat stress conditions. We thereby hypothesized that supplementing heat-stressed dairy cows with L-theanine might affect the concentration and translocation of LPS, as well as the relevant immunoreaction. This study was conducted to explore the effects of L-theanine on the concentrations of LPS within three distinct biofluids (i.e., blood plasma, milk, and rumen fluid) and relevant inflammatory reactions of dairy cattle exposed to heat stress.

2. Materials and methods

2.1. Animals, diets, and management

This experiment was carried out with the permission from the Animal Care Committee (approval number: 20190618), College of Animal Science and Technology, Hunan Agricultural University, Changsha, China. The present trial was conducted from the August to September of 2019 at Deren Dairy Farm (Changde, China). Thirty healthy lactating Chinese Holstein dairy cattle (initial mean ± SE; 462 ± 47 kg body weight, 20 ± 2.4 kg/d milk yield, 189 ± 47 days in milk, and 2 ± 1 parities) were used as experimental animals. In accordance with a completely randomized design, all the cattle were randomly allocated to 1 of 3 dietary treatments: the control (CON), the low L-theanine dosage treatment (LL), and the high L-theanine dosage treatment (HL). Cattle in the CON were provided with a basal total mixed ration (TMR), while cattle in the LL and HL were offered the basal diet top-dressed with 16 or 32 g/d per cow L-theanine, respectively. The L-theanine used in the present trial was a commercial product with a purity >95% (Sunfull Bio-tech Co., Ltd., Changsha, China). This experiment lasted for 38 d, consisting of 7 d of adaption and 31 d for collecting data and samples. All the cattle were housed in a tie-stall barn and offered free access to fresh water. Cows were fed ad libitum twice per day at 05:00 and 17:00, respectively. The ingredients and chemical compositions of the basal TMR are presented in Table 1. Throughout this experiment, the temperature-humidity index (THI) value was obtained using the NRC (1971) formula:

$$\mathrm{THI} = (1.8 \times T_{db} + 32) - (0.55 - 0.0055 \times \mathrm{RH}) \times (1.8 \times T_{db} - 26)$$

where T_{db} represents the dry bulb temperature, while RH means the relative humidity percentage. The THI value was recorded in quadruplicates 3 times per day (08:00, 14:00, and 20:00) through four thermo-hygrometers (9013, Deli Group, Ningbo, China) placed throughout four quadrants of the barn. It was implied that dairy cattle were under heat stress conditions if the THI was above 72 (Bohmanova et al., 2007).

Table 1
Constituents and nutrient composition of the basal TMR.

Item	Content
Ingredients, g/kg DM	
Corn silage	387
Alfalfa silage	71.2
Leymus chinensis hay	10.2
Alfalfa hay	142.5
Oat hay	10.2
Beet pulp	16.3
Whole cottonseed	20.4
Corn meal	134.4
Soybean meal	69.2
Brewer's spent grain	81.5
DDGS	14.3
Wheat bran	20.4
$Ca(HCO_3)_2$	5.1
$NaHCO_3$	5.5
NaCl	1.0
MgO	1.0
Limestone powder	3.7
Premix[1]	6.1
Nutrient content, g/kg DM	
NE_L[2], MJ/kg	6.91
OM	916.9
CP	172.1
NDF	440.2
ADF	286.7
Ether extract	54.7
Ash	83.1
Ca	7.6
P	3.9

TMR = total mixed ration; DDGS = distillers' dried grains with soluble.
[1] Every 1 kg of premix comprises 2,500,000 IU of vitamin A, 100,000 IU of vitamin D_3, 4,000 IU of vitamin E, 500 mg of methionine, 500 mg of lysine, 3,600 mg of Mg, 400 mg of Zn, 350 mg of Mu, 200 mg of Fe, 100 mg of Cu, 96 mg of Cr, 50 mg of Se, and 4.0 mg of Co.
[2] Calculated based on the Cornell-Penn-Miner Dairy System (CPM-Dairy; Boston et al., 2000).

2.2. Sampling

Sample collection was performed using sterilized equipment on d 27 and 38 during this trial, respectively. Four hours after the morning feeding, rumen liquid was obtained from the central rumen using a stomach tube through the oral cavity as previously introduced by Shen et al. (2012). In brief, after discarding the initial 150 mL of rumen liquid, the subsequent 150 mL was adopted and then filtered through 4 layers of gauze under an anaerobic condition. Since dairy cattle were milked twice daily (08:00 and 20:00), samples of milk were collected separately and then pooled at the ratio of 1:1, in accordance with the proportion between each milking yield. To collect the serum samples 4 h after the morning feeding, blood was firstly obtained using vacuum tubes through the tail vein and subsequently placed at room temperature for 30 min, before the final centrifugation was operated at 1,500 × *g* for 10 min at 4 °C. As to the plasma samples, blood was firstly collected using a 10 mL anti-coagulation (heparin sodium) tube, and then centrifuged at 3,500 × *g* for 15 min at 4 °C. All the samples were instantly frozen in liquid nitrogen and stored afterwards at −80 °C until subsequent measurements were conducted.

2.3. Chemical and biochemical analysis

The dry matter (DM; method 930.15), ash (method 942.05), crude protein (method 2001.11), ether extract (method 920.39), neutral

detergent fiber (NDF; method 2002.04), and acid detergent fiber (ADF; method 973.18) in the basal TMR were analyzed in accordance with the procedures of AOAC (2005). The contents of calcium (Ca) and phosphorus (P) in the basal diet were measured using previous methods (Tang et al., 2019; Wang et al., 2020a, 2020b).

The LPS concentrations in the plasma, milk, and rumen fluid were determined through a chromogenic end-point Tachypleus Amebocyte Lysate (TAL) assay kit (EC80545S, Chinese Horseshoe Crab Reagent Manufactory Co. Ltd, Xiamen, China) by referring to the manufacture's instruction and methods introduced precedently (Tao et al., 2014; Wang et al., 2020a, 2020b). In order to ensure that LPS concentrations were in the appropriate range, the dilution for those pretreated samples were carried out as previously described (Wang et al., 2020a, 2020b). The determinations for the LPS-related cytokines (Toll-like receptor [TLR]-4, interleukin [IL]-1β, IL-2, IL-6, and tumor necrosis factor alpha [TNF-α]), and LPS-related acute phase proteins (lipopolysaccharide binding protein [LBP], haptoglobin [Hp] and serum amyloid A [SAA]) in the serum of cattle were conducted in duplicates using corresponding enzyme-linked immunosorbent assay (ELISA) kits (CSB-EL023603BO, CSB-E09810B, CSB-E12899B, CSB-E08585B, CSB-E16370B, CSB-E08592B, CSB-E12020B, Cusabio Technology, Wuhan, China), as depicted by manufacturer's manuals and precedent investigations (Wang et al., 2020a, 2020b; Zhou et al., 2014).

2.4. Statistical analysis

The UNIVARIATE and PLOT procedures were employed to examine the compliance of data gained in this experiment with the assumptions of homogeneity and normality of variances through residual plots (ver. 9.4, SAS Institute Inc.), and non-normally distributed variables were then converted through logarithmic transformation. Data were subsequently analyzed through the PROC MIXED procedure (ver. 9.4, SAS Institute Inc.) to test the effects of supplementing L-theanine. The statistical model involved treatment, sampling date, and their interaction as the fixed effects, with sampling date as the repeated measurement and individual animal as the experimental unit. Also, parity was involved in the statistical model. Least squares means are quoted in the results. Statistical significance was declared at $P < 0.05$, while a trend was discussed at $0.05 < P \leq 0.10$.

3. Results and discussion

Throughout the present experiment, the THI index was recorded in quadruplicates thrice each day and the daily average (mean ± SD) was calculated (Fig. 1). The daily averaged THI value fluctuated from 74 to 83, constantly beyond the heat stress threshold at 72. As a result, it could be deduced that all the dairy cattle were continuously under heat stress conditions during this trial.

In the current study, when compared to the CON, a higher ($P < 0.05$) level of LPS in rumen liquid, but lower ($P < 0.05$) quantities of LPS in plasma and milk were found, as noted in the LL treatment (Table 2). Nevertheless, supplementing the heat-stressed dairy cows with high L-theanine dosage did not significantly influence ($P > 0.05$) the LPS levels across different biofluids compared to the control. Previous investigations have reported the inflammation-alleviating effects of L-theanine in LPS-induced stress in broilers and mice (Li et al., 2018; Wang et al., 2018), and it has been found that L-theanine can improve the intestinal mucosal barrier function through maintaining the mRNA expression levels of tight junction proteins (Wang et al., 2020a, 2020b; Zhang et al., 2020), as well as the concentration of secretory immunoglobulin A (Li et al., 2018). Therefore, it could be inferred that a relatively low dosage of L-theanine might help restore the barrier function of the rumen epithelium during the heat stress of this study, and hence decrease the translocation of LPS through ruminal epithelium and reduce LPS accretion in the milk of dairy cattle. However, due to the limited information on the application of L-theanine in heat-stressed dairy cows, this assumption requires further studies to be verified.

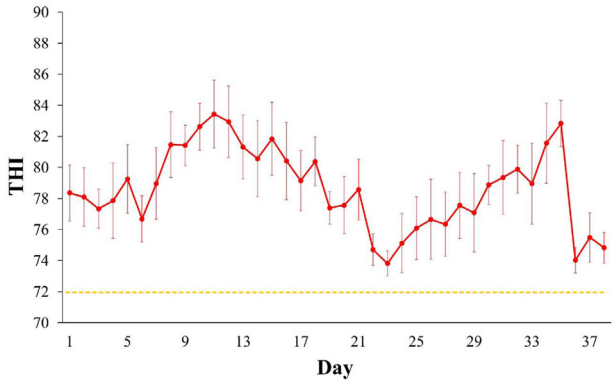

Fig. 1. The daily averaged temperature-humidity index (THI) values throughout this trial.

Table 2
Effects of supplementing L-theanine on LPS concentrations across different biofluids in dairy cows during heat stress.

Item	Biofluid	Treatment			SEM	*P*-value
		CON	LL	HL		
LPS, $\log_{10}$ endotoxin units (EU)/mL	Rumen liquid	4.20^{b}	4.24^{a}	4.22^{ab}	0.012	0.016
	Plasma	3.93^{a}	3.86^{b}	3.89^{ab}	0.021	0.016
	Milk	3.42^{a}	3.29^{b}	3.32^{ab}	0.044	0.021

CON = control; LL = low L-theanine dosage; HL = high L-theanine dosage.
[a, b] Means within a row for treatments which do not have a common letter differ at $P < 0.05$.

The concentrations of these measured inflammatory mediators including acute phase proteins and cytokines in the present trial were similar to the results of our precedent study also conducted in dairy cows under heat stress conditions (Wang et al., 2020a, 2020b). As a specific acute phase protein that interacts with LPS and significantly augments LPS activity, LBP is reckoned as the first biomarker for LPS detection and response of the animal, and its concentration in the circulating blood would hence act as evidence of LPS translocation (Chang et al., 2015; Eckersall and Bell, 2010; Stefanska et al., 2017). Despite the fact that a low dosage of L-theanine reduced LPS concentration in plasma compared to the control in this trial, no significant discrepancy ($P > 0.05$) in the LBP levels within the three treatments was observed (Table 3). This discrepancy between the LPS concentration and LBP reaction could be explained by the sensitivity and activity of LBP and needs to be further investigated. LBP can transfer LPS to the membrane-bound cluster of differentiation antigen 14 (CD14) at cell surfaces, where the LPS-LBP complex interacts with a series of TLR. Further, LPS molecules possessing distinct three-dimensional configurations could interact with different TLR and result in different cytokine inductions and immune reactions (Netea et al., 2002; Plaizier et al., 2012). In this study, neither of the two L-theanine treatments significantly influenced ($P > 0.05$) the TLR-4 level in the serum when compared to the CON. Nonetheless, it was also noted that the concentration of TLR-4 in the HL treatment was significantly lower ($P < 0.05$) than that of the LL treatment. This could be attributed to the possibility that the LPS molecules in the HL group might tend to

Table 3
Effects of supplementing L-theanine on the concentrations of LBP, TLR-4, and relevant pro-inflammatory mediators in the serum of dairy cattle during heat stress.

Item	Treatment			SEM	*P*-value
	CON	LL	HL		
LBP, Log_{10} ng/mL	1.31	1.36	1.18	0.090	0.261
TLR-4, Log_{10} ng/mL	1.26[ab]	1.40[a]	0.80[b]	0.186	0.045
IL-1β, Log_{10} pg/mL	2.35[a]	2.04[b]	1.84[b]	0.129	0.007
IL-2, Log_{10} pg/mL	1.94	1.74	1.66	0.103	0.070
IL-6, Log_{10} pg/mL	1.34	1.36	1.35	0.053	0.872
TNF-α, Log_{10} pg/mL	2.65	2.68	2.66	0.043	0.763
Hp, Log_{10} ng/mL	5.74	5.79	5.80	0.049	0.506
SAA, Log_{10} ng/mL	2.78	2.78	2.72	0.039	0.261

LBP = lipopolysaccharide binding protein; TLR = Toll-like receptor; CON = Control; LL = low L-theanine dosage; HL = high L-theanine dosage; IL = interleukin; TNF-α = tumor necrosis factor alpha; Hp = haptoglobin SAA = serum amyloid A.
[a, b] Means within a row for treatments which do not have a common letter differ at $P < 0.05$.

interact with other TLR rather than TLR-4 (e.g., TLR-1 and TLR-2) and necessitates future studies to be uncovered.

The interaction between LPS-LBP complex and TLR stimulates the secretion of pro-inflammatory cytokines such as IL-1β, IL-2, IL-6 and TNF-α (Wang et al., 2018; Ye et al., 2016). It was marked in the current trial that supplementing L-theanine at both low and high levels significantly reduced ($P < 0.05$) the level of IL-1β, whilst tended ($0.05 < P \leq 0.10$) to reduce the generation of IL-2 when compared with the control, implying that the dietary supplement of L-theanine could remit the inflammation response of dairy cattle under heat stress conditions. The levels of TNF-α and IL-6 in the serum were unaffected ($P > 0.05$) by supplementing L-theanine, which might be due to the disparities in the activity across cytokines and/or the LPS profiles in this experiment.

The generation of those aforementioned pro-inflammatory cytokines normally causes the acute phase response. This response comprises the secretion of acute phase proteins primarily by hepatocytes, among which Hp and SAA are the most reactive in cows (Alsemgeest et al., 1994; Samarasinghe et al., 2020). In this trial, no significant ($P > 0.05$) variations in the concentrations of Hp and SAA within the serum within three groups were noticed. This result could be attributed to the sensitivity of acute phase proteins to inflammatory stimulation and/or the LPS profiles in the dairy cattle of this study (Jacobsen et al., 2004; Stefanska et al., 2017).

4. Conclusion

In summary, supplementing dairy cows with L-theanine at 16 g/d per cow under heat stress conditions decreased the flux of LPS from the rumen into peripheral blood and reduced LPS concentration in the milk. Furthermore, the level of IL-1β in the serum was lowered in response to the addition of L-theanine at both 16 and 32 g/d per cow during heat stress. Consequently, it could be deduced that L-theanine could serve as a promising feed additive in mitigating the detrimental effects of heat stress on dairy cows, and its supplementation at a relatively low dosage (16 g/d per cow) could achieve better effects in terms of preventing endotoxemia and relieving relevant inflammatory responses. Future investigations are required to interpret the discrepancies in the responses of LPS and inflammatory mediators to the supplementation of L-theanine, as well as the mechanisms in the inflammation alleviation of L-theanine in dairy cattle exposed to heat stress.

Author contributions

Zuo Wang: Conceptualization, Methodology, Formal analysis, Investigation, Visualization, Writing – original draft, Project administration, Funding acquisition. **Lingmei Zhang:** Investigation, Visualization, Project administration. **Yuannian Yu:** Investigation, Project administration. **Lingyuan Yang:** Project administration. **Peihua Zhang:** Project administration. **Weijun Shen:** Project administration. **Fachun Wan:** Project administration. **Jianhua He:** Project administration. **Wenjun Xiao:** Resources. **Zhicai Li:** Resources, Project administration. **Dasheng Yang:** Project administration. **Zhiliang Tan:** Supervision, Funding acquisition. **Shaoxun Tang:** Conceptualization, Methodology, Formal analysis, Investigation, Visualization, Supervision, Project administration, Funding acquisition.

Conflict of 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.

Acknowledgements

The present study received the funding through the Hunan Provincial Natural Science Foundation (Grant No. 2019JJ50279, 2019RS3021), Hunan Provincial Education Department (Grant No. 19B257), Hunan Provincial Science and Technology Department (Grant No. 2017NK1020), Ministry of Science and Technology of China (Grant No. 2018YFD0501604), and National Natural Science Foundation of China (Grant No. 31772633).

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