Protective effects of zymosan on heat stress-induced immunosuppression and apoptosis in dairy cows and peripheral blood mononuclear cells

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Abstract

Dairy cows exposed to heat stress (HS) show decreased performance and immunity, but increased heat shock protein expressions and apoptosis. Zymosan, an extract from yeast cell walls, has been shown to modulate immune responses and defense against oxidative stress. However, few literatures are available about the effects of zymosan on immune responses and other parameters of the dairy cows under HS. Here, both primary peripheral blood mononuclear cell (PBMC) and dairy cow models were established to assess the effects of zymosan on performance, immunity, heat shock protein, and apoptosis-related gene expressions of dairy cows under HS. In vitro study showed that proliferation, IL-2 production, and *Bcl-2/Bax-α* ratio of cow primary PBMC were reduced, whereas *hsp70* mRNA and protein expressions, as well as Annexin V-binding, were increased when PBMCs were exposed to heat. In contrast, zymosan significantly reversed these above changes induced by the HS. In the in vivo study, 40 Holstein dairy cows were randomly selected and assigned into zymosan group (supplemental zymosan; $n = 20$) and control group (no supplemental zymosan; $n = 20$). The results showed that zymosan improved significantly the dry matter intake and milk yield, increased IgA, IL-2, and tumor necrosis factor- α (TNF- α) contents in sera, as well as hepatic *Bcl-2/Bax-α* ratio, but decreased respiration rate and hepatic *hsp70* expressions in the dairy cows under HS. Taken together, zymosan could alleviate HS-induced immunosuppression and apoptosis and improve significantly the productive performance and immunity of dairy cows under HS.

Keywords Zymosan · Heat stress · Dairy cows · Peripheral blood mononuclear cells · Immunity · Apoptosis

Introduction

Heat stress (HS) is one of the most important environmental stressors for dairy cows. Previous studies have demonstrated that HS not only reduced the productive and reproductive

performances (Kadokawa et al. 2012) but also depressed immune function of dairy cows (Carroll et al. 2012; Tao et al. 2012). On the one hand, HS significantly affected innate immunity of dairy cows as measured by the neutrophil function (Do Amaral et al. 2011), lymphocyte proliferation (Elvinger et al. 1991), dysregulation of cytokine expression, and even induced immunosuppression (Ju et al. 2014; Nakamura et al. 2001). On the other hand, HS also affected humoral immunity of calves by decreasing immunoglobulin contents in the blood (Kelley et al. 1982), even reduced the ability of the new-born calf to acquire adaptive immunity (Dahl et al. 2016; Monteiro et al. 2014). Furthermore, a recent study indicated that heat stress induced apoptosis in dairy cows through the Bax/Bcl-2 pathway (Li et al. 2016). Therefore, it is necessary to develop more effective strategies to protect dairy cows from HS damage.

Our previous study found that addition of yeast culture to the diet could improve the productive performance, energy status, and heat shock protein (*hsp*) mRNA level in

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lymphocytes of dairy cows exposed to heat (Liu et al. 2014). Zymosan, a yeast-derived β -glucan- and mannan-rich particle (Li et al. 2013), is the main component of yeast culture. Meanwhile, zymosan is a natural antioxidant (Kogan et al. 2005) and modulates innate and adaptive immune responses (Jiang et al. 2013; Song et al. 2015). In addition, cows could better withstand HS due to the higher survivability of the lymphocytes (Bhanuprakash et al. 2016).

Therefore, in this study, both primary PBMC and dairy cow models were established to assess the effects of zymosan on performance, immunity, heat shock protein, and apoptosis-related gene expressions of dairy cows under HS.

Materials and methods

Zymosan

Purified zymosan (Z4250, Sigma-Aldrich, USA) was dissolved in phosphate-buffered saline (PBS), heated in water bath at 95 °C for 1 h following ultrasonic modification for 2 h, and then autoclaved to prepare a stock solution (5 mg/ml). Subsequently, the solution was packaged and stored at –20 °C for in vitro study. The stock solution was diluted to the desired concentration for further experiments using a serum-free RPMI-1640 medium (Invitrogen, USA). As a feed additive, zymosan, provided by Angel Yeast Co., Ltd. (Yichang, Hubei, China), includes β -D-glucan (> 20%), mannan-oligosaccharides (30 to 40%), and protein (< 35%). For usage, it was mixed into the total mix ration (TMR) at the mass ratio of 1:1000.

Ethics statement

This research protocol was approved by the Ethics Committee for Animal Experimentation of Nanjing Agricultural University (SYXK (Su) 2011-0036). All animal care and use procedures were conducted in strict accordance with the Animal Research Committee guidelines of the College of Veterinary Medicine at Nanjing Agricultural University.

Isolation and culture of cow primary PBMCs

During winter months, ten healthy lactating Holstein cows with similar body score and milk yield were randomly selected. The blood samples (10 ml/cow) were taken from the caudal vein of each cow into a vacuum tube containing EDTA-K2 before the morning feeding and milking. Subsequently, primary PBMCs were immediately isolated from the EDTA blood samples by density gradient centrifugation as previously described with slight modifications (Lacetera et al. 2005; Kishore et al. 2014). Briefly, the whole blood cells diluted in PBS were gently layered over LTS-1077 (Tianjin Haoyang

Biological Manufacture Co., Ltd., China) and centrifuged. The mononuclear cell band (white cloudiness) was harvested and washed with PBS. Residual red blood cells were eliminated by hypotonic shock treatment using redistilled water. Isolated PBMCs were suspended in RPMI-1640 medium (Invitrogen, USA), containing 10% fetal calf serum with 1% streptomycin and penicillin. Cells were counted using a hemocytometer and 0.4% trypan blue dye exclusion, and stored at a concentration of 5×10^6 /ml.

Cell viability determination by MTT and LDH

Primary PBMCs (5×10^5 cells/well) were seeded into 96-well plates and then incubated with various concentrations of zymosan (0–2000 μ g/ml) for 48 h. During the final 4 h, the PBMCs were subjected to the colorimetric 3-(4,5-dimethylthiazol-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay (Sigma, USA). Finally, the optical density (OD) value was determined using a Multimode Reader (Bio-Rad, USA) as the absorbance at 490 nm with a secondary wavelength of 655 nm.

Lactate dehydrogenase (LDH) release was measured using commercially available kits. Briefly, PBMCs were seeded in 96-well plates and exposed to various concentrations of zymosan (0–2000 μ g/ml). After 48-h incubation, the medium was collected into 1.5-ml EP tubes and centrifuged (12,000 $\times$ g, 4 °C) for 15 min. The supernatants were collected to determine LDH release according to the manufacturer's protocol (Jiancheng, China). The absorbance was measured at a wavelength of 450 nm.

Determinations of PBMC proliferation and IL-2 release by MTT and ELISA

Primary PBMCs (5×10^5 cells/well) were seeded into two 96-well plates and then incubated with 0, 10, 30, 60, and 90 μ g/ml of zymosan in the absence or presence of phytohemagglutinin (PHA), respectively. After 24-h incubation, these two plates were incubated at 37 and 42 °C (5% CO₂) for 3 h, respectively, then allowed to recover at 37 °C (5% CO₂) for 21 h. During the final 4 h, the PBMCs were subjected to the MTT assay. Finally, the OD value was determined as above described.

Similar to above described, after total 48-h incubation, the supernatants were collected to determine interleukin-2 (IL-2) release according to the manufacturer's protocol for the IL-2 enzyme-linked immunosorbent assay (ELISA) kit (Jiancheng, China).

Apoptosis assay by flow cytometry

Primary PBMCs were seeded in 6-well plates and incubated with 0, 10, 30, 60, and 90 μ g/ml of zymosan, respectively.

After 24-h incubation, heat treatment was conducted as above described. After total 48-h incubation, cells were collected and stained with Annexin v-FITC and/or PI staining buffer (Vazyme, China) according to the manufacturer's instructions and then prepared for flow cytometry. Cells were shocked slightly and analyzed with a FACSCalibur flow cytometer (BD Biosciences, USA) and CellQuest Pro software (BD Biosciences, USA). Data were analyzed using FlowJo for Mac 10.7 (Tree Star, Inc.).

Animals and experiment design

In total of 40 healthy lactating Holstein cows (parity 3.3 ± 0.5 ; 60 ± 15 days in milk; 650 ± 54 kg body weight; average milk yield of 33.2 ± 1.7 kg/day) were randomly selected from a commercial dairy farm in Nanjing, Jiangsu, China ($32^{\circ}0' \text{ N}$, $118^{\circ}8' \text{ E}$, and altitude 8.9 m). All cows had access to fresh water ad libitum and were individually fed a TMR two times daily at 0700 and 1900 hours to allow 5% feed refusals. The TMR was formulated based on the NRC (2001) guidelines for lactating Holstein cows, and its ingredient was presented as previously described by our co-authors without any modifications (Liu et al. 2014). All cows fed with the same basal diet were acclimated for 1 week before the onset of feeding trial.

During summer months, 40 cows were randomly assigned into zymosan group (supplemental zymosan; $n = 20$) and control group (no supplemental zymosan; $n = 20$). The supplemental zymosan was individually hand mixed into the TMR at the mass ratio of 1/1000 daily. This study was performed for 28 days, during which time the air temperature (AT, $^{\circ}\text{C}$) and relative humidity (RH, %) were recorded three times daily at 0700, 1400, and 2100 h to calculate the temperature-humidity index (THI) according to the equation: $\text{THI} = (0.8 \times \text{AT}) + [\text{RH} \times (\text{AT} - 14.4)] + 46.4$. AT was presented in Fig. S1, and the mean daily THI varied from 74.8 to 86.2 (HS threshold: $\text{THI} > 72$) (Brown et al. 2016). All cows were milked two times daily at 0800 and 2000 hours, and milk yield and composition were measured on days 1, 7, 14, and 28 (Table 1). Rectal temperature and respiration rate were also measured and recorded weekly (Table 2).

Sample collection and measurement

For each cow, 20 ml blood sample was collected from the caudal vein before morning feeding and milking on days 1, 7, 14, and 28. The blood samples were allowed to clot at room temperature (RT) for 2 h, and the sera were separated by centrifugation ($1500 \times g$, 4°C) for 20 min and stored at -80°C until analysis. The serum IgA, IgG, IgM, IL-1, IL-2, IL-6, and tumor necrosis factor- α (TNF- α) contents were measured using the corresponding ELISA kits (Jiancheng, China).

Liver tissue samples were taken on day 28 as previously described (Xu et al. 2016). Briefly, all cows were treated with

Table 1 Dry matter intake, milk yield, and composition of 40 heat-stress cows fed basal (control) and zymosan supplemental diets

Group	Control	Zymosan
DMI, kg/day	$19.2 \pm 0.2^{\text{A}}$	$20.5 \pm 0.3^{\text{B}}$
Milk yield, kg/day	$29.4 \pm 1.2^{\text{A}}$	$34.2 \pm 1.5^{\text{B}}$
Milk lactose, kg/day	1.52 ± 0.05	1.48 ± 0.05
Milk lactose, %	5.06 ± 0.12	4.98 ± 0.12
Milk fat, kg/day	0.96 ± 0.08	1.02 ± 0.04
Milk fat, %	3.21 ± 0.07	3.37 ± 0.05
Milk protein, kg/day	0.86 ± 0.04	0.91 ± 0.04
Milk protein, %	3.05 ± 0.06	3.03 ± 0.05

Means within the row with different letters are significantly different, $P < 0.01$

DMI dry matter intake

local anesthesia (subcutaneous injection of procaine between their 11th or 12th right intercostal space), and then 30–50 mg liver tissues (hepatic lobe depth in 3–5 cm) were sampled from this space by a biopsy needle (6 mm in diameter) according to the vacuum principle. Finally, liver tissues were immediately washed in ice-cold 0.9% saline solution, placed in micro-centrifuge tubes, and then frozen at -80°C for quantitative real-time PCR (qRT-PCR) analysis later.

SYBR qRT-PCR analysis

Total RNA was extracted from both frozen liver tissues and PBMCs using the RNAiso Plus reagent (TaKaRa, China), and cDNA was synthesized according to the manufacturer's protocol (TaKaRa, China). The PCR primers were designed using the Primer Premier 5.0 program (Premier Biosoft International, USA), and the individual primer sequences are presented in Table 3. The PCR reactions were conducted using the StepOnePlus™ Real-Time PCR System (Applied Biosystems, USA) as previously described (Zhuang et al. 2015). Briefly, 25 μl of the reaction mixture contains 12.5 μl of $2 \times$ UltraSYBR Mixture (TaKaRa, China), 2 μl of cDNA, 0.5 μl of forward primer (10 μM), 0.5 μl of reverse primer

Table 2 Rectal temperature and respiration rate of 40 heat-stress cows fed basal (control) and zymosan supplemental diets

Group	Control	Zymosan
Rectal temperature, $^{\circ}\text{C}$		
0630 hours	39.0 ± 0.1	38.9 ± 0.1
1830 hours	39.4 ± 0.2	39.3 ± 0.2
Respiration rate, times/min		
0630 hours	56.5 ± 1.4	55.8 ± 1.4
1830 hours	$75.6 \pm 2.4^{\text{A}}$	$68.8 \pm 2.3^{\text{B}}$

Means within the row with different letters are significantly different, $P < 0.01$

Table 3 Primers used for quantitative real-time PCR

Gene	Accession no.	Primer sequences (5'-3')	Product size/bp
<i>β-Actin</i>	AY141970.1	F: GCAGGTCATCACCATCGG R: CCGTGTGGCGTAGAGGT	158
<i>HSP27</i>	BT021550	F: CACTCGCAAATACACGCTGC R: GTTTTCCGGCTGTTCCGACT	199
<i>HSP70</i>	BTU09861	F: CGGCTTAGTCCGTGAGAACA R: CCGCTCGGTATCGGTGAA	289
<i>HSP90</i>	NM_001079637	F: CCAAGTCTGGCACTAAAG R: GAAGACTCCCAAGCATAC	163
<i>Bcl-2</i>	U92434	F: CTTCGCCGAGATGTCCAG R: CTACCCAGCCTCCGTTGT	257
<i>Bax-α</i>	U92569	F: GCAAACCTGGTGCTCAAGG R: GCACTCCAGCCACAAGA R: ATGGGTACGGGCAAAA	189

HSP27 heat shock protein 27, *HSP70* heat shock protein 70, *HSP90* heat shock protein 90

(10 μM), and 9.5 μl of RNase-free dH₂O. The thermal cycling conditions consisted of 10 min at 95 °C, 15 s at 95 °C (40 cycles), and 1 min at 60 °C, followed by 15 s at 60 °C. Each reaction was performed in triplicate, and a negative control with no cDNA template was used as a calibrator. The relative expression levels of the target genes were calculated using the $2^{-\Delta\Delta CT}$ method with *β-actin* as an endogenous reference gene as described previously (Gan et al. 2015).

Western blot analysis

PBMCs were washed twice with PBS and harvested into lysis buffer (60 μl per well of a six-well plate) containing protease inhibitor (Beyotime, China) for total protein extraction. The protein concentration was measured using a BCA kit (Beyotime, China). To avoid protein degradation, 60 μg of protein was diluted in mercaptoethanol and loading buffer at 95 °C for 5 min to denature. Subsequently, the denatured protein was subjected to 10 to 15% SDS-PAGE and transferred electrophoretically to PVDF membranes. The membranes were blocked for 2 h at RT with Tris-buffered saline Tween-20 (TBST) containing 5% bovine serum albumin (BSA) and 0.1% Tween 20, followed by overnight incubation with primary antibodies including anti-HSP70 antibody (5A5), anti-Bcl-2 antibody (100/D5), anti-Bax-α antibody (2D2), and anti-*β-actin* antibody (ACTN05(C4)) from abcam, UK, at 4 °C. After three washes, the membranes were incubated with appropriate secondary antibodies (horseradish peroxidase labeled anti-mouse secondary antibody, Cell Signaling Technology, USA) at RT for 1 h. Finally, the blots were visualized with an enhanced chemiluminescence system (Bio-Rad, USA).

Statistical analysis

Statistical analysis was conducted using Prism 6 (GraphPad Software, La Jolla, CA). The Kolmogorov-Smirnov test was

used to test for normal distribution of the variables. Normal distribution was considered at $P > 0.10$. Data were presented as mean ± SEM. The unpaired two-tailed Student's *t* test for two-group comparisons, one-way ANOVA with Tukey post-test, and two-way ANOVA with Dunnett's post-test for multigroup comparisons were performed to evaluate statistical significance. Statistical significance was considered at $P < 0.05$.

Results

Cytotoxic effects of zymosan on primary cultured PBMCs

In the in vitro study, MTT and LDH assays were preliminarily conducted to evaluate the effects of various concentrations of zymosan on PBMC viabilities for the purpose of excluding any toxic effects of zymosan. As present in Fig. 1a, b, zymosan was shown with a significant toxic effect at the concentration of up to 250 μg/ml, and there were no obviously toxic effects when the concentration of zymosan was less than 125 μg/ml. Therefore, zymosan at the concentrations of 10, 30, 60, and 90 μg/ml was selected for the subsequent experiments.

Zymosan alleviated the HS-induced immunosuppression in primary cultured PBMCs

PBMC proliferation and IL-2 production were determined to assess the effects of HS on immune responses. As shown in Fig. 2a, PBMC proliferation significantly decreased at 42 °C compared with that at 37 °C, which indicated that the proliferation of PBMC was suppressed when exposed to heat. The IL-2 production was extremely low without the stimulation of PHA both at 37 and 42 °C but increased sharply with PHA

Fig. 1 Cytotoxic effects of zymosan on primary cultured PBMCs. Primary PBMCs were seeded in 96-well plates and exposed to zymosan at 0, 15.625, 31.25, 62.5, 125, 250, 500, 1000, and 2000 μg/ml to measure **a** cell viability and **b** LDH release by MTT and LDH. Data are

presented as mean ± SEM from three independent experiments. Statistical significance was considered at $P < 0.05$, compared with no zymosan; * $P < 0.05$ and ** $P < 0.01$

stimulation. Moreover, IL-2 production was lower at 42 °C than that of 37 °C, suggesting that the exposure of PBMCs to the heat suppressed the secretion of IL-2.

However, the suppression of PBMC proliferation was significantly alleviated by 30–90 μg/ml of zymosan. Furthermore, with an increase in the zymosan concentration, PBMC proliferation enhanced and reached a maximum with zymosan at 60 μg/ml. As expected, IL-2 production significantly increased in PBMCs at 42 °C when zymosan was at 90 μg/ml, which indicates that zymosan could alleviate the HS-induced suppression of IL-2 production in PBMCs. Taken together, these data suggest that zymosan could alleviate the HS-induced immunosuppression.

Zymosan alleviated the HS-induced hsp70 overexpression and apoptosis in primary cultured PBMCs

Hsp70 family was regarded as a reliable and sensitive biomarker for hyperthermia and the management of HS in large farm animals (Mishra et al. 2011; Modrow et al. 2012; Rout et

al. 2016). A previous study indicated that hsp70 was able to inhibit apoptosis to protect the body from damage (Mosser et al. 1997). To further assess the effects of zymosan on HS, hsp70 expression and apoptosis were determined using qRT-PCR, western blotting, and flow cytometry. As shown in Fig. 3a, *hsp70* mRNA in primary PBMCs exposed to the heat significantly increased, and the increase in *hsp70* mRNA was down-regulated significantly by 30 to 90 μg/ml of zymosan. Meanwhile, with an increase in zymosan concentration, the levels of *hsp70* mRNA and its protein in heat-treated PBMCs decreased in a dose-dependent manner, and the maximal effects were observed with zymosan at 90 μg/ml (Fig. 3a, c). The *Bcl-2/Bax-α* mRNA ratio was lower at 42 °C than that at 37 °C (Fig. 3b). On the contrary, with the increase of zymosan concentration, the ratio increased significantly in a dose-dependent manner and reached a peak when zymosan was at 90 μg/ml. In addition, western blot analysis showed that zymosan significantly increased Bcl-2 but decreased Bax-α expressions (Fig. 3c), and resulted in a marked increase of Bcl-2/Bax-α protein ratio, suggesting that HS-induced apoptosis was alleviated by zymosan. Furthermore, the flow cytometry

Fig. 2 Zymosan alleviates HS-induced immunosuppression in primary cultured PBMCs. Primary PBMCs were treated with various concentrations of zymosan in the absence or presence of PHA for 48 h at 37 and 42 °C, respectively. **a** PBMC proliferation was measured by MTT. **b** IL-2

production was measured using a commercial assay kit. Data are expressed as mean ± SEM from three independent experiments. Within different temperatures, bars with * $P < 0.05$ and with ** $P < 0.01$. Within the same temperature, bars without a common letter differ, $P < 0.05$

Fig. 3 Zymosan alleviates HS-induced hsp70 overexpression and apoptosis in primary cultured PBMCs. Primary PBMCs were seeded in 12-well plates and cultured with various concentrations of zymosan for 48 h at 37 and 42 °C, respectively. **a**, **b** Quantitative real-time PCR was performed to determine the relative *hsp70*, *Bcl-2*, and *Bax-α* mRNA. Within different temperatures, bars with ** $P < 0.01$. Within the same temperature, bars without a common letter differ, $P < 0.05$. **c** Total proteins were extracted and subjected to western blot assay. The expression levels of

hsp70, *Bcl-2*, and *Bax-α* were presented as mean $\pm$ SEM of triplicate samples after background corrections. * $P < 0.05$ and ** $P < 0.01$ compared with no zymosan. **d** Flow cytometry assay for the detection of apoptosis. The total apoptosis rate was equal to the sum of late apoptosis rate (Q2) and early apoptosis rate (Q3). Data are presented as mean $\pm$ SEM from three independent experiments. ** $P < 0.01$ compared with no zymosan

analysis (Fig. 3d) demonstrated the apoptosis rate of PBMCs significantly decreased with the increase of zymosan concentration, supporting the above results. Taken together, zymosan could alleviate HS-induced hsp70 overexpression and apoptosis in primary PBMCs.

Zymosan improved the productive performance of dairy cows under HS

To further investigate the effects of supplemental zymosan on the productive performance of dairy cows under HS, dry matter intake (DMI), milk yield and composition, and rectal temperature and respiration rate were recorded and analyzed. As shown in Tables 1 and 2, compared with the control group, cows fed with zymosan improved dramatically DMI and milk yield, and reduced significantly the respiration rate, but no obvious changes were seen in other parameters. Taken

together, our results suggested that zymosan could improve the productive performance of dairy cows under HS.

Zymosan improved immunity of dairy cows under HS

To further verify the in vitro results, the serum IgA, IgG, IgM, IL-1, IL-2, IL-6, and TNF- α contents were determined to assess the immunity of dairy cows under HS in vivo. Compared with the control group, cows fed with zymosan had increased significantly IgA concentration in sera, but no obvious changes were observed in the concentrations of IgG and IgM (Fig. 4a). These findings indicated that zymosan might activate mucosal immunity mediated by IgA to improve humoral immunity of dairy cows under HS. The effects of zymosan on the levels of IL-1, IL-2, IL-6, and TNF- α are shown in Fig. 4b. There was a significant increase in IL-2 and TNF- α in dairy cows from zymosan

Fig. 4 Effects of zymosan on the levels of immunoglobulins and inflammatory cytokines in sera. Blood samples were collected from every cow on days 1, 7, 14, and 28, and the levels of **a** immunoglobulins and **b** inflammatory cytokines in sera were

determined in triplicate. Data are presented as mean $\pm$ SEM from three independent experiments. Groups were compared with a two-way ANOVA followed by Dunnett's post-test. * $P < 0.05$ and ** $P < 0.01$ compared with control. ns no significance

group, relative to that from control group, but no obvious changes were seen in the concentrations of IL-1 and IL-6. Taken together, these data supported the in vitro results, suggesting that zymosan could, at least, partly improve immunity of dairy cows under HS.

Zymosan alleviated *hsp70* overexpression and apoptosis in dairy cows under HS

To further verify the in vitro results, the *hsp*- and apoptosis-related mRNA in livers of dairy cows was determined. As shown in Fig. 5a, zymosan significantly reduced *hsp70* mRNA in livers of dairy cows under HS compared to control, but no obvious changes were seen in the levels of *hsp27* and *hsp90* mRNA. In addition, compared with control, zymosan significantly increased *Bcl-2* but decreased *Bax-α* mRNA expressions in the liver tissues from dairy cows under HS (Fig. 5b). These data supported the in vitro results, suggesting that

zymosan could alleviate *hsp70* overexpression and apoptosis in dairy cows under HS.

Discussion

Yeast culture had been used as a nutritive supplement in feed of dairy cows and goats for defense against HS (Shwartz et al. 2009; Wang et al. 2016). In addition, our previous study indicated that the supplement of yeast culture could improve performance in dairy cows under HS (Liu et al. 2014). However, no study has focused on the effects of zymosan, the main ingredient of yeast cell walls, on the dairy cows under HS. In the present study, both in vivo and in vitro studies were performed to investigate productive performance, immunity, HSPs, and apoptosis in dairy cows.

In this study, supplemental zymosan improved dramatically DMI and milk yield and reduced significantly the respiration

Fig. 5 Effects of zymosan on *hsp*- and apoptosis-related mRNA levels in livers. Liver tissues were taken from every cow on day 28. The relative **a** *hsp*- and **b** apoptosis-related gene mRNA levels were measured by quantitative real-time PCR. Each PCR reaction was performed in triplicate.

Data are presented as mean $\pm$ SEM of triplicate experiments. The unpaired two-tailed Student's *t* test for two-group comparisons was performed to evaluate statistical significance. * $P < 0.05$ and ** $P < 0.01$ compared with control. ns no significance

rate in dairy cows under HS, suggesting that zymosan could improve productive performance of dairy cows under HS. This finding is very important given that HS often affected the health and biological functions of dairy cows through decreasing milk production and reproductive performance (Polsky and von Keyserlingk 2017), even negatively affected milk yield of dry cows in the subsequent lactation (Fabris et al. 2017). Moreover, a previous study confirmed that HS abatement in the late gestation period improved performance of dairy cows in subsequent lactation (Karimi et al. 2015). So, zymosan supplement may be used as a heat abatement strategy in the future.

HS affected humoral immunity through decreasing serum IgG content (Kelley et al. 1982). However, our results showed that serum IgA content in zymosan group significantly increased, but the contents of IgG and IgM did not present obvious changes compared with that in control group. Zymosan could activate mucosal immunity mediated by IgA, improving humoral immunity of dairy cows under HS. However, the action mechanism of zymosan on mucosal immunity needed further study. Hepatic and intestinal blood flows are reduced during hyperthermia to accelerate the body heat dispersion, and the intestinal immunity played an important role in defense of HS (Deja et al. 2010; Quinteiro-Filho et al. 2010). In general, the intestinal mucosal barrier includes a mucosal mechanical barrier, a biological barrier and an immunological barrier, and the immunological barrier is formed by secretory IgA. In addition, Liu et al. (2012) reported that HS could damage intestinal mucosal immune function by reducing serum IgA (Liu et al. 2012). So, it is likely that zymosan could alleviate the immunity of dairy cows under HS via improving the intestinal mucosal immune function.

The PBMC proliferation and cytokine productions were assessed to further understand the immune status in dairy cows under HS in this study. Previous studies have shown that lymphocyte proliferation was reduced following exposure to 42 °C (De Rensis et al. 2015; Ju et al. 2014), which supports the observations in this study. In addition, zymosan (10 to 90 µg/ml) blocked the decrease in the proliferation of PBMCs exposed to heat. Our novel finding may be a pathway to activate innate immunity in dairy cows under HS given that zymosan is a kind of immuno-stimulant (Ross et al. 1985). As an important sign of T cell activation (Carter et al. 1998), IL-2 production was also affected by HS. In this study, IL-2 production was lower in PBMCs exposed to 42 than 37 °C, which confirmed that HS suppressed the secretion of IL-2 supporting the previous study (Sherwood et al. 1987). This study showed that zymosan significantly increased IL-2 production in PBMCs exposed to heat, suggesting that zymosan was able to alleviate the HS-induced suppression of IL-2 production. Correspondingly, zymosan also significantly increased IL-2 and TNF- α in sera from dairy cows under HS. All results indicated that zymosan could promote cytokine secretion to alleviate immunosuppression induced by HS, which might be

responsible for the effects of zymosan as an immunostimulant (Song et al. 2015). Furthermore, zymosan, a known toll-like receptor 2 (TLR2) agonist (Sato et al. 2003), plays a vital role in regulation of immune responses via TLR2 (Frasnelli et al. 2005). Growing evidence indicates that zymosan plays a protective effect on multiple immune cells via inducing TLR2 (Du et al. 2017; Taghavi et al. 2018). So, we infer that zymosan may alleviate heat stress-induced immunosuppression via activating TLR2 signaling.

In this study, the levels of apoptosis-related mRNA in livers and PBMCs were analyzed. Overall, the *Bcl-2/Bax- α* was significantly decreased in PBMCs exposed to the heat, indicating that HS induced cell apoptosis, which is consistent with the previous results (Sellins and Cohen 1991; Sodja et al. 1998). However, with the treatment of zymosan, the *Bcl-2/Bax- α* in the heat-treated PBMCs and livers significantly increased, and Annexin V-binding in the heat-treated PBMCs significantly decreased. These results were novel findings, suggesting that zymosan could reduce HS-induced apoptosis in vitro and in vivo. In addition, previous studies have indicated that *hsp70* was also able to inhibit apoptosis to protect the body from damage (Mosser et al. 1997), and the *hsp70* family was regarded as a reliable and sensitive biomarker for hyperthermia and the management of HS in large farm animals (Mishra et al. 2011; Modrow et al. 2012; Rout et al. 2016). In this study, the *hsp70* mRNA and protein in PBMCs exposed to 42 °C significantly increased, which was consistent with the previous results (Mishra et al. 2011), and the changes were reversed by zymosan, which suggested that zymosan indeed alleviated HS. In general, *hsp70* expression in tissues under HS increases to protect polypeptides against denaturation and aggregation, and then return to basal levels when HS is attenuated. Zymosan attenuates HS and induces inflammation, thereby leading to immune response homeostasis. Zymosan may down-regulate *hsp70* via inducing immune response homeostasis. Abnormal proteins increase when the cells respond to environmental stress and act as substrates to bind competitively to *hsp70* resulting in breakup of *hsp70*-heat shock transcription factor (HSF) complex (Radons 2016). Specially, ATP decreases when the cells are under HS, resulting in non-separation of substrate from *hsp70* thereby promoting *hsp70*-HSF breakup and initiating more expression of *hsp70*. In turn, when the HS is attenuated by zymosan or free *hsp70* and ATP are abundant, the abnormal proteins (substrates) decrease and *hsp70*-HSF complexes reform, thereby inhibiting *hsp70* expression. Hence, we infer that *hsp70* (ATP-dependent chaperones) (Mymrikov et al. 2017) substrates may increase and then decrease in the thermal treated cells/tissues with differences in how various *hsp70* proteins recognize their respective substrates (Umehara et al. 2018; Zhang et al. 2014).

In conclusion, zymosan could improve significantly the productive performance and immunity of dairy cows under HS and alleviate HS-induced *hsp70* overexpression and apoptosis.

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Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interests.

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