J. Dairy Sci. 108:1978–1991
https://doi.org/10.3168/jds.2024-25133

Effects of milk extracellular vesicles from Holstein Friesian and Brown Swiss heat-stressed dairy cows on bovine mammary epithelial cells

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ABSTRACT

The increase in ambient temperature is responsible for a behavioral, physiological, and metabolic responses known as heat stress, which affects dairy cows' general well-being, health, reproduction, and productivity. Focusing on the functioning of the mammary gland, attention has been recently paid to a new method of cell-cell communication mediated by extracellular vesicles, which with their cargo can affect the target cells' phenotypic traits, behavior, and biological functions. This study investigated whether the small extracellular vesicles (sEV) isolated from milk of heat-stressed Holstein Friesian (H) and Brown Swiss (B) cows affect the cellular response of a bovine mammary epithelial cell line (BME-UV1). To this purpose, 8 mid lactation cows, 4 of each breed fed the same diet and kept in the same barn, which experienced the same hyperthermia during a natural heat wave, were chosen to collect 2 milk different samples: under thermoneutrality (TN, d1) and under heat stress (HS, d 8) conditions. The sEV were isolated from skim milk samples through differential centrifugations, characterized for size and concentration by nanoparticle tracking analysis. Integrity of the milk sEV membranes was evaluated by transmission electron microscopy and presence of EV markers through western blotting. Then BME-UV1 cells were incubated for 24 h with different pooled milk sEVs (H-TN, H-HS, B-TN, B-HS). Cell viability and apoptosis assay, reactive oxygen species production, and mRNA expression of heat shock proteins and antioxidant genes by reverse transcription and real time PCR were determined. In vivo results showed an increase in rectal temperature and respiration rate, a reduction in milk yield both for H and B dairy cows, with a lowest decrease observed in B cows compared with H cows. In vitro results of BME-UV1 cells treated with milk sEV H-HS and B-HS showed an alteration of the cell viability and metabolic activity, by reducing or increasing reactive oxygen species accumulation, and suppressing or increasing the expression of stress-associated genes thereby modulating the response of BME-UV1 according to the animals' thermal condition and the breed. These findings indicated that the small vesicles of Brown milk triggered cellular defense against heat stress, supporting the Brown Swiss breed's thermotolerance.

Key words: heat stress, Holstein Friesian, Brown Swiss, extracellular vesicles, bovine mammary epithelial cells-UV1

INTRODUCTION

The increase in ambient temperature triggers a physiological and metabolic responses known as heat stress (**HS**) in animals and, particularly in dairy cows, affecting the general well-being, health, reproduction, and productivity (Bernabucci et al., 2010; Polsky and von Keyserlingk, 2017; Liu et al., 2019). The milk yield reduction is the most recognized effect during HS (Bernabucci et al., 2010; Tao et al., 2020), where a fluctuation is observed in milk production in different stages of lactation (Rhoads et al., 2009; Basiricò et al., 2016; Tao et al., 2018). Moreover, HS adversely affects mammary gland functions (Hao et al., 2018; Tao et al., 2018, 2020; Yue et al., 2020; Skibiel et al., 2022).

The degree to which HS causes hyperthermia and other changes in physiological function in dairy cows is determined partly by genetics (Cuellar et al., 2023). Depending on the anatomical and physiological characteristics of the species or breed, there may be different levels of thermoregulatory responses (Gatenby, 1986; Bligh, 1998; Gebremedhin et al., 2008). Milk production of Holstein Friesian cows significantly decreases during HS (Bernabucci et al., 2014; Gantner et al., 2017; Jurkovich et al., 2023). In contrast, the effects of HS on milk yield are more limited in breeds that are not highly productive. Maggiolino et al. (2020) observed no temperature-

Received May 8, 2024.
Accepted October 24, 2024.
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The list of standard abbreviations for JDS is available at adsa.org/jds-abbreviations-24. Nonstandard abbreviations are available in the Notes.

Table 1. Characteristics of the 2 groups at the beginning of the experimental period. Data are expressed as means ± SD (n = 4 for both Holstein and Brown Swiss breed)

Breed	Number of lactations	DIM	BCS	Number of observations
Holstein	2.75 ± 1.48	105.75 ± 7.69	2.39 ± 0.19	4
Brown Swiss	3.00 ± 1.58	103.00 ± 6.20	2.69 ± 0.18	4
P-value	0.82	0.60	0.06	

humidity index (**THI**) thresholds for milk production in Brown Swiss dairy cows. This different thermal tolerance of breeds, due to the negative genetic relationship between heat tolerance and productive traits (Bohlouli et al., 2013; Nguyen et al., 2017), could be used to select animals with greater tolerance to HS and high productivity (Bernabucci et al., 2010).

To further investigate the possible changes and mechanisms occurring in the bovine mammary gland during HS, a method of cell-to-cell communication mediated by extracellular vesicles (**EV**) has been investigated. Small EV (**sEV**; Welsh et al., 2024) are 30 to 100 nm in diameter, surrounded by a lipid bilayer, are of endosomal derivation (Zhang et al., 2019) with a density of 1.13 to 12.19 g/mL (Schorey et al., 2015), and contain molecular information (proteins, lipids, mRNA, miRNAs, and DNA) derived from the original cell (Zempleni et al., 2017). Small EV are present in most cells and biofluids (Whiteside, 2018; Yagi et al., 2017; Han et al., 2018), suggesting that they are an evolutionarily conserved cell-to-cell communication mechanism (Schorey et al., 2015). Thanks to their cellular cargo, sEV can influence cellular processes, and phenotypic characteristics of target cells (Simons and Raposo, 2009; Zhang et al., 2019), exerting different effects depending on the cell of origin and pathophysiological conditions (Tkach and Théry, 2016). Over the last decade, the number of studies on milk-derived EV has grown exponentially (Admyre et al., 2007; Mecocci et al., 2022). Recently, attention has been addressed to milk sEV (**mEV**) from cows (Hata et al., 2010; Izumi et al., 2015; Blans et al., 2017; Colitti et al., 2020). It has been observed that mEV content differs between species (Ngu et al., 2022) and is also involved in the HS response process in animals (Gebremedhn et al., 2020). The origin of EV has not been fully elucidated, although suggested to be secreted by mammary epithelium cells and immune cells (Admyre et al., 2007; Melnik et al., 2014; van Herwijnen et al., 2016). Blans et al. (2017) characterized the lipid profiles of bovine mEV demonstrating an enrichment in sphingomyelin and phosphatidylethanolamine and, at lower concentrations, phosphatidylserine and phosphatidylcholine, which promote membrane integrity and stability in body fluids exerting beneficial effects. Moreover, the proteome of mEV has been investigated showing that the mEV transport proteins with anti-inflammatory and antimicrobial activities (Samuel et al., 2017; Yang et al., 2017).

The miRNA cargoes of mEV could be considered a promising approach to studying the molecular mechanism underlying the host responses to mastitis (Stefanon et al., 2023) and may affect the calf intestine development and immune response (Van Hese et al., 2020). Menjivar et al. (2023) reported that sEV of granulosa cells mitigate the effect of HS on bovine oocytes and embryos in vitro. To date, the effect of mEV on the mammary gland epithelium has not been investigated so far. Therefore, this study aimed at investigating whether the mEV isolated from milk of heat-stressed Holstein Friesian and Brown Swiss lactating cows differently affect the cellular response of an in vitro experimental model of bovine mammary epithelial cell line (**BME-UV1**).

MATERIALS AND METHODS

Ethics Statement

All animal use and procedures for the study were approved by the Ethical Committee for Animal Welfare of Animals employed in scientific research of the Department of Veterinary Medicine of the University of Bari (approval no. 05/2022).

Animals and Environmental Conditions

The trial was carried out on 8 mid-lactating cows, 4 Italian Holstein Friesian (**H**) and 4 Brown Swiss (**B**), which experienced the same hyperthermia during a natural heat wave. The H and B cows were selected from 30 multiparous mid-lactating cows for each group raised on the same commercial farm (Azienda Bruna Nuova di Maellaro, Noci, Italy; 40°43′53.4″N 17°06′50.1″E). The 2 groups of cows were balanced for parity, DIM, milk yield level, and BCS (Table 1). For the trial, the dairy cattle were fed the same diet (Table 2) in the same barn. The experimental period started on July 18, 2022, and lasted 8 d. To monitor the microclimatic conditions of the barn, 5 data loggers (Hobo Pro series Temp probes, Onset Computer Corp., Pocasset, MA) for the hourly recording of environmental temperature and relative humidity have been placed in the barn according to barn orientation, shading, solar radiations, and air movement. Data have been stored and managed through a remote data collection system. During the experimental period,

air temperature and relative humidity were monitored; dairy cows' rectal temperature (**RT**), and respiration rate (**RR**), were registered in the morning (4 a.m.), afternoon (3 p.m.), and evening (8 p.m.). Two milk samples were taken from each animal at the morning milking (4 a.m.) and afternoon milking (3 p.m.) and milk fat and protein contents were assessed. The temperature and relative humidity dataset obtained by data loggers was used for calculating the THI according to the formula reported by Bernabucci et al. (2014):

$$THI = (1.8 \times AT + 43) - (0.55 - 0.55 \times RH) \times [(1.8 \times AT + 32) - 58].$$

Energy-corrected milk and fat- and protein-corrected milk (**FPCM**) were calculated using the following formulas (Gaines and Dadison, 1923; Yan et al., 2011):

$$ECM = (12.95 \times \text{Fat yield}) + (7.65 \times \text{Protein yield}) + (0.327 \times \text{milk yield})$$

$$FPCM = \text{milk (kg/d)} \times (0.1226 \times \text{Fat \%} + 0.0776 \times \text{Protein \%} + 0.2534)$$

Table 2. Composition of the standard diet

Item	Total
Feed,[1] kg/head	
Multigrass meadows hay	10.50
Mixed feed[2]	7.85
Corn meal	7.00
Wheat flour middling	0.30
Sugar cane molasses	0.30
Nutrients, % on DM basis	
DM	88.40
CP	15.69
aNDFom[3]	38.52
ADF	22.08
Ether extract	3.13
Ash	6.52
Starch	24.82

[1]Wet basis.

[2]Composition: extruded whole soybean, toasted and extruded soybean meal, barley flour, cotton seed, hulled sunflower flour, wheat flour, wheat bran, corn gluten feed, calcium carbonate, sodium chloride, dicalcium phosphate, calcium and magnesium carbonate, magnesium oxide, sodium bicarbonate, magnesium sulfate. Supplements per kg: vitamin A 54,000 UI; vitamin D_3 6,000 UI; vitamin E 180 mg; choline chloride 270 mg; niacin 270 mg; betaine 60 mg; biotin 0.24 mg; calcium D pantothenate 6 mg; vitamin B_1 6.0 mg; vitamin B_2 6.0 mg; vitamin B_{12} 0.06 mg; vitamin B_6 3.0 mg; trace elements: Fe (iron carbonate) 34.44 mg; Fe (iron chelate) 6.13 mg; Mn 120.0 mg; I (potassium iodide) 1.26 mg; Cu (copper chelate of glycine-solid hydrate) 28.2 mg; Se (sodium selenite) 0.6 mg; Zn (zinc sulfate monohydrate) 178.8 mg; bentonite 6,000 mg.

[3]Neutral detergent fiber analyses corrected for residual ash content.

EV Isolation

The milk samples from all the cows, collected on d 1 during the morning milking (4 a.m.) and on d 8 in the afternoon milking (3 p.m.), were immediately skimmed by centrifugation at 2,800 × *g* for 30 min at 4°C and used to isolate the mEV (Figure 1). The mEV were isolated from skim milk samples (12 mL each) of 4 H and 4 B dairy cows collected under thermoneutrality (**TN**, d 1) and HS (d 8) conditions. Milk was centrifuged at 10,000 × *g* for 30 min at 4°C to remove the remaining fat and cellular debris. The supernatant was collected, diluted in triple-filtered (0.22 µ*M*) sterile PBS, and ultracentrifuged at 100,000 × *g* for 1 h at 4°C using a fixed rotor (Beckman Coulter TY65 fixed angle rotor, Pasadena, CA). The mEV pellet (2 mL) was collected and further purified through size exclusion chromatography (**SEC**) using the qEVOriginal 35 nm columns from Izon (Izon Science, Oxford, UK), following the manufacturer's instructions, as previously reported (Ávila Morales et al., 2023). A mix of mEV by pooling the same proportion of mEV (1:4) of each condition (TN or HS) for the 2 breeds (H and B) was created; these mixes were used for all in vitro cellular assays. Then, mEV were depleted from LPS with ToxinEraser Endotoxin Removal Kit (GenScript, Piscataway, NJ) according to the manufacturer's instructions. Collected mEV were stored at −80°C for further in vitro assays.

Characterization of the Size Distribution of mEV

The size and concentration of mEV were assessed by nanoparticle tracking analysis (**NTA**) using the NanoSight LM10-HS system (Amesbury, UK). Five 30-s recordings were made for each sample. The collected data were analyzed with NTA software, which provides high-resolution particle-size distribution profiles and concentration measurements of the EV (particles/mL).

Transmission Electron Microscopy

To distinguish single EV and evaluate the integrity of the membranes, mEV (2.5µL) were loaded on glow-discharged carbon-coated formvar copper grids, negatively stained with 2% uranyl acetate, air-dried for 10 min, and observed in an FEI Talos 120kV transmission electron microscope (FEI Company, the Netherlands). Images of mEV were acquired by a 4k × 4k Ceta complementary metal-oxide semiconductor (CMOS) camera (Thermo Fisher Scientific, Waltham, MA) camera.

Identification of sEV Marker Proteins by Western Blot

Western blot analysis was performed to evaluate the presence of EV markers (Supplemental Figure S1, see

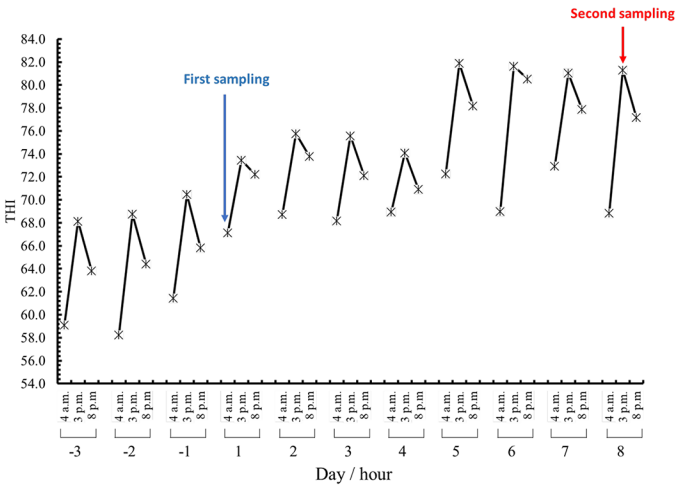

Figure 1. Temperature-humidity index (THI) during the experimental period of 8 d and description of milk sample collection for small extracellular vesicles isolation.

Notes). Four micrograms of total proteins were loaded. Following SDS-PAGE, proteins were transferred to nitrocellulose membranes using Trans-Blot Turbo Midi 0.2 µm Nitrocellulose Transfer Packs (Biorad Laboratories s.r.l., Milano, Italy) on the Trans-Blot Turbo Transfer System (Biorad). The membrane was blocked for 1 h with ROTIBlock 1X (Carl Roth, Schoemperlenstr 3–5, D-76185 Karlsruhe, Germany; catalog no. A151.1), incubated with primary antibodies anti-CD9 (Biorad, MCA469GT, 1:1,500) overnight at 4°C and then with the secondary antibody polyclonal anti-mouse peroxidase (Dako, Agilent, P0260, 1:2,000) for 1 h at room temperature, or with the primary antibody anti-TSG101 (Abcam, Cambridge, United Kingdom, ab225877, 1:2,000) overnight at 4°C and secondary antibody polyclonal anti-rabbit peroxidase (Vector Laboratories, PI-1000, 1:3,000) for 1 h at room temperature. Immunoreactive bands were visualized by enhanced chemiluminescence (Millipore, Merk Life Science S.r.l., Milano, Italia).

Cell Lines Condition

The BME-UV1 (RRID: CVCL_W716) cells were used as a model to replicate the biology of the bovine mammary gland tissue (Zavizion et al., 1996). This clonal cell line presents a morphology typical of luminal epithelial cells, and it was used to investigate mammary metabolism, involution, and apoptosis (Arévalo Turrubiarte et al., 2016; Basiricò et al., 2019). The BME-UV1 cell line was obtained from primary bovine mammary epithelial cells cultivated by stable transfection with SV-40 large T-antigen. Cells were seeded into 75 cm^2 culture flasks with a culture medium. The basal growth medium was constituted of Dulbecco's Modified Eagle Medium/Nutriente mixture F-12, Roswell Park Memorial Institute (RPMI) 1640 medium, and NCTC 135 medium (a chemically defined medium developed by the National Cancer Institute) in a 5:3:2 ratio, supplemented with 10% exosome-depleted fetal bovine serum (**dFBS**) Qualified One Shot (Gibco, Life Technologies Corporation, New York), 0.1% lactose, 0.1% lactalbumin hydrolysate, 1.2 m*M* glutathione, 1 µg/mL insulin, 5 µg/mL transferrin, 1 µg/mL hydrocortisone, 0.5 µg/mL progesterone, 10 µg/mL ascorbic acid, and antibiotics (penicillin 100 IU/mL; streptomycin 100 µg/mL). All medium supplements, except fetal bovine serum Qualified One Shot, were from Sigma-Aldrich. The cells were maintained at 37°C in a humidified 5% CO_2 incubator until confluence. The BME-UV1 cells were resuspended in a complete culture medium to a concentration of 2×10^6 cells/flask. After 24 h, the medium was removed and replaced with a complete culture medium enriched with different pooled mEV (H-TN, H-HS, B-TN, B-HS). All experiments using pooled mEV were repeated 3 times, with 3 technical replicates for each experiment.

Cell Viability Assays

The BME-UV1 cells were seeded at 2.5×10^4 cells/well in 96 microplates using dFBS. After 24 h, BME-UV1 cells were challenged with 200 mEV/cell of different pooled mEV (H-TN, H-HS, B-TN, B-HS) and incubated for 24 h at 37°C. The cell viability was determined using the Cell Proliferation Kit II (Roche Applied Science, Indianapolis, IN) according to the manufacturer's instructions. The 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2h-tetrazolium-5-carboxanilide (**XTT**) cell viability assay was based on the dehydrogenase activity in the active mitochondria of cells. For each well, 50 µL of XTT labeling reagent and electron coupling reagent (50:1) were added at 37°C. After 24 h, absorbance was measured at 450 nm and 650 nm. Background absorbance was subtracted from each value.

Apoptosis Assay

The Apo-ONE Homogeneous Caspase-3/7 Assay kit (Promega, Madison, WI) was used to detect the apoptosis in BME-UV1 cells treated with different pooled mEV (H-TN, H-HS, B-TN, B-HS) according to the manufacturer's instructions. The assay was based on the measurement of caspase-3 and -7 activities in cells. For each treatment, 2.5×10^4 BME-UV1 cells/well were seeded on 96 microplates using dFBS and incubated at 37°C. After 24 h, the medium was replaced with medium enriched with different pooled mEV (H-TN, H-HS, B-TN, B-HS), cells were incubated for 24 h and then 100 µL of a mixture of Apo-ONE caspase 3/7 reagent and Apo-ONE

Table 3. DNA sequences of bovine forward and reverse primer used for real-time PCR analysis

Gene[1]	Primer	Temperature of annealing (°C)	Accession No.
GAPDH forward	CCCAGAATATCATCCCTGCT	60	NM_001034034.2
GAPDH reverse	CTGCTTCACCACCTTCTTGA		
18S forward	CGCAGCTAGGAATAATGGAA	60	NR_036642.1
18S reverse	TCTGATCGTCTTCGAACCTC		
HSPA1A forward	AGGACTTCGACAACAGGCTG	59	NM_203322.3
HSPA1A reverse	TGCTGGACGACAAGGTTCTC		
HSP90AA1 forward	TCACTGAGGAAATGCCACCC	60	NM_001012670
HSP90AA1 reverse	ATGGAGACAGAGCGCTGAAC		
GRP94 forward	TGCTGTGTGGAGAGGGAATG	60	NM_174700.2
GRP94 reverse	TCCTGTGACCACAATCCCAA		
GRP78 forward	TGCGAAGCCCTATAGCTGAC	60	NM_001075148.1
GRP78 reverse	AGTAGGTGGTACCCAGGTCG		
SOD1 forward	CGAGGCAAAGGGAGATACAG	59	NM_174615.2
SOD1 reverse	CAATATCCACGATGGCAACA		
SOD2 forward	TCAATAAGGAGCAGGGACGC	59	NM_201527.2
SOD2 reverse	AAGCCGTGTATCGTGCAGTT		

[1]*GAPDH* = glyceraldehyde-3-phosphate dehydrogenase, *18S* = *18S* ribosomal RNA, *HSPA1A* = heat shock protein family A (HSP70) member 1A; *HSP90AA1* = heat shock protein 90 α family class A member 1; *GRP94* = glucose-regulated protein 94; *GRP78* = glucose-regulated protein 78; *SOD1* = superoxide dismutase 1; *SOD2* = superoxide dismutase 2.

Buffer (1:10) were added in each well. The samples are stirred with a plate shaker for 5 s. Following incubation at room temperature for 30 min, caspase 3/7 activity was estimated from the fluorescence to 485/535 nm with Multimode Detector DTX 880 (Beckman Coulter Inc., Indianapolis, IN).

Detection of Intracellular Reactive Oxygen Species

Reactive oxygen species (**ROS**) production was determined by 2′,7′-dichlorodihydrofluorescein diacetate probe (**DCFH-DA**), seeding cells in 96 microplates at a density of 2.5×10^4 cells/well, as described above. After 24 h, the medium of BME-UV1 cells was replaced with a medium enriched with different pooled mEV (H-TN, H-HS, B-TN, B-HS). After 24 h, the cells were washed twice with PBS and incubated with 20 µ*M* DCFH-DA at 37°C. Forty minutes later, the fluorescence intensity was measured using a fluorescence microplate reader (Multimode Detector DTX 880, Beckman Coulter Inc., Indianapolis, IN) at an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

Effects of Different mEV on BME-UV1 Gene Expression

The possible effects of mEV isolated from H and B in TN and HS conditions on mRNA expression of BME-UV1 cells were determined by real-time PCR. For each mEV treatment, the BME-UV1 cells were cultivated at the concentration of 2×10^6 in a complete medium and challenged with different pooled mEV as described above. Briefly, the total RNA was extracted with an RNA Concentrator kit (A&A Biotechnology, Gdynia, Poland) according to the manufacturer's protocol and stored at −80°C. The RNA was quantified using a Quan-It kit (Invitrogen, Waltham, MA) and fluorescence was measured at wavelengths of 590/670 nm with Multimode Detector DTX 880 (Beckman Coulter Inc., Indianapolis, IN). Total RNA (100 ng) was reverse transcribed using the SensiFAST cDNA Synthesis Kit (Bioline Reagents, London, UK) in a final volume of 20 µL on a PCR Express thermal cycler (Hybaid, Ashford, UK). The real-time quantitative PCR analysis was performed in the LyghtCycler 2.0 (Roche Applied Sciences, Indianapolis, IN) using SYBR Green detection following the manufacturer's instructions. The heat shock genes involved in heat stress response (*HSPA1A* = heat shock protein family A [HSP70] member 1A; *HSP90AA1* = heat shock protein 90 α family class A member 1; *GRP94* = glucose-regulated protein 94; *GRP78* = glucose-regulated protein 7; Saeed-Zidane et al., 2017; Gebremedhn et al., 2020; Yue et al., 2020), and genes involved in defense systems of oxidative damage response (*SOD1* = superoxide dismutase 1; Primer-3Plus online design platform, https://www.primer3plus.com/index.html, and *SOD2* = superoxide dismutase 2; Khan et al., 2020) were investigated. The primers used for the real-time PCR are reported in Table 3. The specific primer pairs were verified with Primer-Blast online (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi, November 2023) to confirm their targeted specificity. To account for possible variation related to cDNA input or the presence of PCR inhibitors, each gene was simultaneously quantified for all samples. The glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and *18S* rRNA genes were included as reference genes to normal-

Table 4. Rectal temperature (RT), respiration rate (RR), milk yield, milk fat, milk proteins, ECM, and fat- and protein-corrected milk (FPCM) in Holstein and Brown Swiss dairy cows under thermoneutral (TN) and heat stress (HS) conditions; data are expressed as means ± SD

Breed	EC[1]	THI[2]	RT, °C	RR, breaths/min	Milk yield, L/cow	Milk fat, %	Milk proteins, %	ECM L/cow	FPCM, L/cow
Holstein	TN	67.1	38.30 ± 0.54[a]	58.86 ± 13.41[a]	19.58 ± 0.38[b]	4.02 ± 0.61[a]	3.28 ± 0.17[b]	21.50 ± 1.52[b]	19.59 ± 1.44[b]
	HS	81.3	40.16 ± 0.42[b]	101.71 ± 13.55[b]	14.10 ± 3.21[a]	4.20 ± 0.76[b]	3.10 ± 0.27[a]	15.71 ± 4.04[a]	14.30 ± 3.69[a]
Brown Swiss	TN	67.1	38.18 ± 0.26[a]	40.50 ± 10.35[a]	18.68 ± 2.65[b]	4.70 ± 0.12[b]	3.48 ± 0.21[b]	22.42 ± 2.98[b]	20.50 ± 2.71[b]
	HS	81.3	39.28 ± 0.60[b]	98.88 ± 7.70[b]	16.03 ± 0.72[a]	4.40 ± 0.58[a]	3.33 ± 0.17[a]	18.49 ± 2.03[a]	16.88 ± 1.91[a]

[a,b]Mean values in the same row with different superscripts differ ($P < 0.05$) for confronting of TN condition and HS condition within breed.
[1]EC = environmental conditions.
[2]THI = temperature-humidity index.

ize the relative expression of target genes and the mRNA expression levels of the target genes were calculated using the $2^{-\Delta\Delta CT}$ comparative threshold cycle (**CT**) method (Livak and Schmittgen, 2001).

Statistical Analysis

Analysis of variance model was used to test for a significant effect of environmental conditions (low THI and high THI) within the breed (H and B) on RT, RR, milk yield, fat, proteins, ECM, and FPCM measured parameters using XLSTAT Software 2023 (Lumivero, Denver, CO). The cell viability assays, apoptosis assay, ROS assay, and gene expression were subjected to a normality test. The Shapiro-Wilk test was used to determine the residuals distribution and the data analyzed had a normality residuals distribution. After, these data were analyzed using a 2-tailed *t*-test using XLSTAT Software 2023 (Lumivero). The significance was declared at $P < 0.05$.

RESULTS

Environmental Conditions, Physiological, and Production Parameters in H and B Dairy Cows Under TN and HS Conditions

Data on THI, RT, RR, milk yield, milk fat, milk protein, ECM, and FPCM recorded during the experimental period are shown in Table 4. The THI (Figure 1) has been used to describe the microclimate conditions. Day 1 represented the TN condition (THI = 67.1); d 8 represented the HS condition (THI = 81.3). During the trial, from d1 to d8, changes in RT, RR, milk fat, milk protein, and milk yield were observed due to the heat wave on both breeds. The RT and RR were greater ($P < 0.05$) in H-HS and B-HS conditions compared with TN conditions. In particular, the RT and RR were 40.16°C and 101.71 breaths/min in H-HS and 39.28°C and 98.88 breaths/min in B-HS, respectively. The daily milk yield (**MY**) was reduced ($P < 0.05$) under the HS condition in both breeds of −5.48 and −2.65 L/cows compared with the MY registered under TN condition in H and B, respectively. An increase in milk fat content ($P < 0.05$) and a decrease in milk protein content ($P < 0.05$) in H-HS cows compared with H-TN (0.20% and −0.18%, respectively) were observed. Milk fat and milk protein contents were both reduced ($P < 0.05$) in B-HS compared with B-TN (−0.20% and −0.15%, respectively). The daily ECM and FPCM were decreased ($P < 0.05$) in H-HS and B-HS compared with TN conditions (−5.79 and −5.29 L/cow in H and −3.93 and −3.62 L/cow in B for ECM and FPCM, respectively).

EV Characterization

The mEV isolated from H and B in TN and HS conditions were isolated by ultracentrifugation and SEC. The particle-size distribution of mEV isolated from 3 H and 3 B in TN and HS was assessed by NTA, revealing that the mEV population was characterized by small vesicles. The modal average of the H cows was 123.6 nm ± 10.99 nm in TN and 131.6 nm ± 21.8 nm in HS; in B cows it was 119.23 nm ± 37.88 nm during TN and 127.73 nm ± 13.7 nm during HS. The mEV concentration was 6.41E+10 (SEM 1.81E+09) and 8.65E+10 (SEM 4.10+09) particles/mL in H during TN and HS, respectively. In B, mEV were concentrated at 1.62E+10 (SEM 2.93E+09) and 1.5E+10 (SEM 1.66E+09) particles/mL during TN and HS, respectively. No statistical differences were identified in size or concentration. The shape and integrity of mEV were assessed by electronic transmission microscopy, showing the presence of whole, undamaged mEV (Figure 2A–D). Western blot analysis was performed to examine the expression of different markers in mEV of TN and HS cows. Two different markers, one expressed on the membrane of the EV, the tetraspanin CD9, and the other delivered in the lumen of EV, the TSG101, were identified (Figure 2E).

HS-mEV Reduced BME-UV1 Cell Viability Without Affecting Apoptosis

Figure 3 shows the different effects of mEV treatments (H-TN, H-HS, B-TN, B-HS) on BME-UV1 viability. Milk sEV isolated from milk collected during the heat

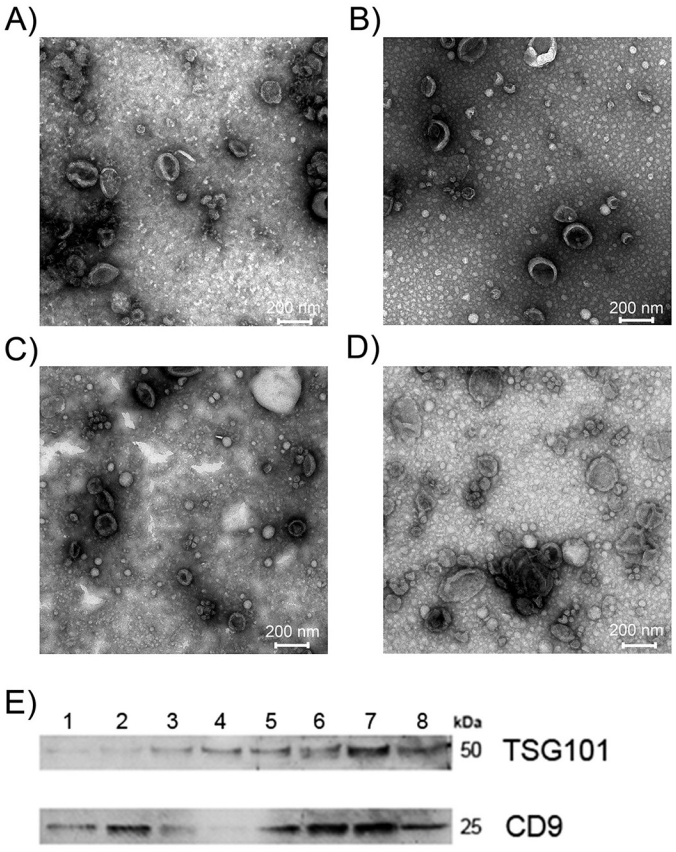

Figure 2. Transmission electron microscope images of milk extracellular vesicles (mEV) isolated from: (A) Holstein cows during TN; (B) Holstein cows during HS; (C) Brown Swiss cows during TN; and (D) Brown Swiss cows during HS. (E) Western blot images showing the presence of markers on the membrane of the EV, the tetraspanin CD9, and in the lumen of EV, the TSG101. Lines 1 and 3: mEV isolated from Holstein cows during TN; lines 2 and 4: mEV isolated from Holstein cows during HS; lines 5 and 7: mEV isolated from Brown Swiss cows during TN; lines 6 and 8: mEV isolated from Brown Swiss cows during HS.

wave reduced cell viability compared with mEV isolated in TN. In detail, mEV H-HS (Figure 3A) and mEV B-HS (Figure 3B) treatments reduced the cell viability and the metabolic activity of BME-UV1 cells compared with cells treated with mEV H-TN and mEV B-TN by −2.80% ($P < 0.05$) and −3.53% ($P < 0.01$), respectively.

The apoptotic activity of BME-UV1 cells was not affected by the coculture with mEV (TN and HS) isolated from the 2 breeds (H and B; Figure 3C and 3D).

ROS Production in BME-UV1 After Different mEV Treatments

Intracellular ROS (Figure 4) production was assessed in BME-UV1 cells after coculture with different mEV treatments (H-TN; H-HS; B-TN; B-HS). Milk sEV isolated from H and B exerted an opposite effect on BME-UV1 cells. In detail, H-HS induced a reduction (−8.0%) of intracellular ROS levels in BME-UV1 cells compared with H-TN cells ($P < 0.05$; Figure 4A). In contrast, an increase ($P < 0.05$; Figure 4B) in the level of intracellular ROS by 12.39% was observed when comparing cells treated with mEV B-HS versus B-TN.

Expression of Genes Related to the HS and Oxidative Stress Response in BME-UV1 Cocultured with Different mEV

To determine the ability of mEV isolated from H and B obtained under TN and HS conditions to modulate the response of BME-UV1 cells, the mRNA expression of genes involved in HS cellular response was evaluated. The gene expression of 2 cytoplasmatic (*HSPA1A*; *HSP90AA1*) and 2 endoplasmic reticulum (*GRP78* and *GRP94*) genes involved in HS (Figure 5) and the expression of 2 antioxidant genes (*SOD1* and *SOD2*; Figure 6) were quantified. The gene expression of *HSPA1A* was downregulated ($P < 0.01$; Figure 5A) in mEV H-HS treated cells compared with mEVs H-TN treated cells (fold change between the HS condition and the TN condition; $FC_{HS/TN}$ −0.33). In contrast, *HSPA1A* mRNA level was upregulated ($P < 0.01$; Figure 5B) in mEV B-HS treated cells compared with mEV B-TN treatment ($FC_{HS/TN}$ +0.63). The *HSP90AA1* gene expression was downregulated ($P < 0.01$; Figure 5C) in mEV H-HS BME-UV1 treated cells compared with mEV H-TN ($FC_{HS/TN}$ −0.50). Instead, the mRNA level of the *HSP90AA1* gene was upregulated ($P < 0.01$; Figure 5D) in mEV B-HS treated cells compared with mEV B-TN ($FC_{HS/TN}$ +0.86). The expression of the *GRP78* gene in mEV H-HS treated cells was downregulated ($P < 0.01$; Figure 5E) compared with mEV H-TN treated cells ($FC_{HS/TN}$ −0.54). Conversely, in mEV B-HS treated cells, the gene expression of *GRP78* was upregulated ($P < 0.01$; Figure 5F) compared with mEV B-TN treated cells ($FC_{HS/TN}$ +0.37). Lastly, the mRNA level of *GRP94* was downregulated ($P < 0.01$; Figure 5G) in mEV H-HS treated cells ($FC_{HS/TN}$ −0.42), and the mRNA level of *GRP94* was upregulated ($P < 0.01$; Figure 5H) in mEV B-HS treated cells compared with mEV B-TN treted cells ($FC_{HS/TN}$ +0.24). The gene expression of *SOD1* did not show any differences ($P > 0.05$; Figure 6A) between mEV H-HS and H-TN treated cells; in contrast, the mRNA level of *SOD1* was upregulated ($P < 0.01$; Figure 6B) in mEV B-HS treated cells ($FC_{HS/TN}$ +0.82) compared with cells treated with mEV B-TN. The expression of the *SOD2* gene was downregulated ($P < 0.05$; Figure 6C) in mEV H-HS treated cells ($FC_{HS/TN}$ −0.15) compared with mEV H-TN treated cells. On the contrary, the mRNA level of *SOD2* was upregulated ($P < 0.01$; Figure 6D) in mEV B-HS treated cells ($FC_{HS/TN}$ +0.67) than in cells treated with mEV B-TN.

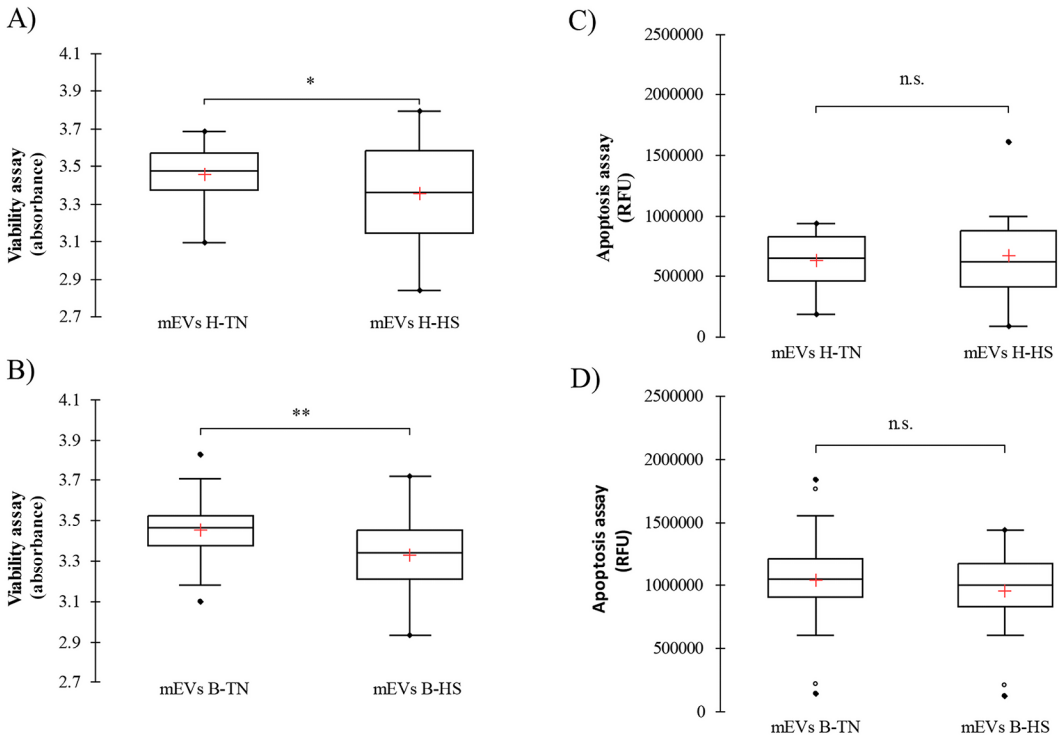

Figure 3. Cell viability and apoptosis assay of bovine mammary epithelial cells (BME-UV1) after 24 h of coculture with different types of milk small extracellular vesicles (mEV) in 2 different breeds and environmental conditions. (A) Cell viability of cells treated with mEV of Holstein (H) cows in thermoneutral (TN) and heat stress (HS) conditions. (B) Cell viability of cells treated with mEV of Brown Swiss (B) cows in TN and HS conditions. Data are presented as the percentages (%) of means relative to mEV TN conditions ± SE. Significant differences among TN and HS treatments of mEV were reported (*$P < 0.05$; **$P < 0.01$). (C) Apoptosis assay of cells treated with mEV of Holstein (H) cows in TN and HS conditions. (D) Apoptosis assay of cells treated with mEVs of Brown Swiss (B) cows in TN and HS conditions. Data are presented as the relative fluorescence units (RFU) of means relative to mEV TN conditions ± SE (n = 9); n.s. = not significant = $P > 0.05$. Midlines represent the median of the data; red crosses represent the mean of the data; dots represent outliers; lower edges of boxes represents the first quartile (Q1); upper edges of boxes represent the third quartile (Q3); and whiskers indicate the variability of the data outside the central 50%.

DISCUSSION

In the current study, the in vivo trial confirmed that warm temperatures with THI above 80 affect physiological variables (RT and RR), MY, fat and protein content, ECM, and FPCM in both breeds. Values of RT and RR clearly show that both breeds were exposed to TN and HS conditions during the first and second sampling, respectively. Both H and B dairy cows showed an increase in RT and RR and a reduction in milk yield and milk

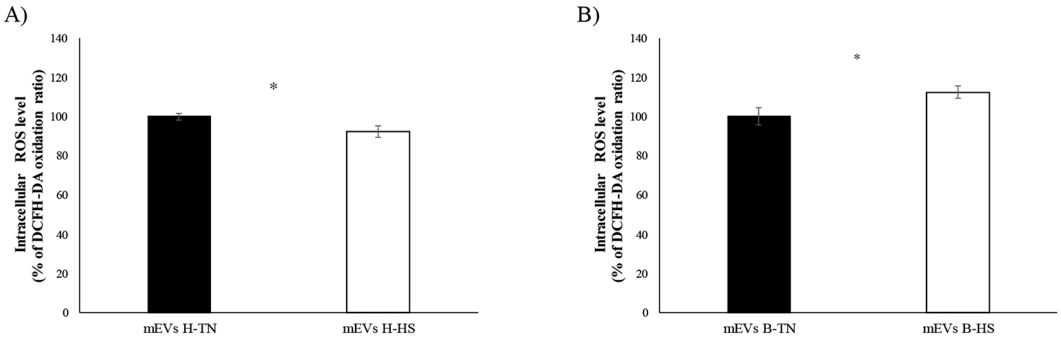

Figure 4. Intracellular ROS level in bovine mammary gland (BME-UV1) cells after 24 h of coculture with different types of milk small extracellular vesicles (mEV) in 2 different breeds and environmental conditions. (A) Intracellular ROS level of cells cocultured with mEV of Holstein (H) cows in thermoneutral (TN) and heat stress (HS) conditions. (B) Intracellular ROS level of cells treated with mEVs of Brown Swiss (B) cows in TN and HS conditions. Data are presented as the percentage (%) of means DCFH-DA oxidation ratio relative to TN conditions ± SE (n = 9). Significant differences between mEV TN and mEV HS treatments were reported (*$P < 0.05$).

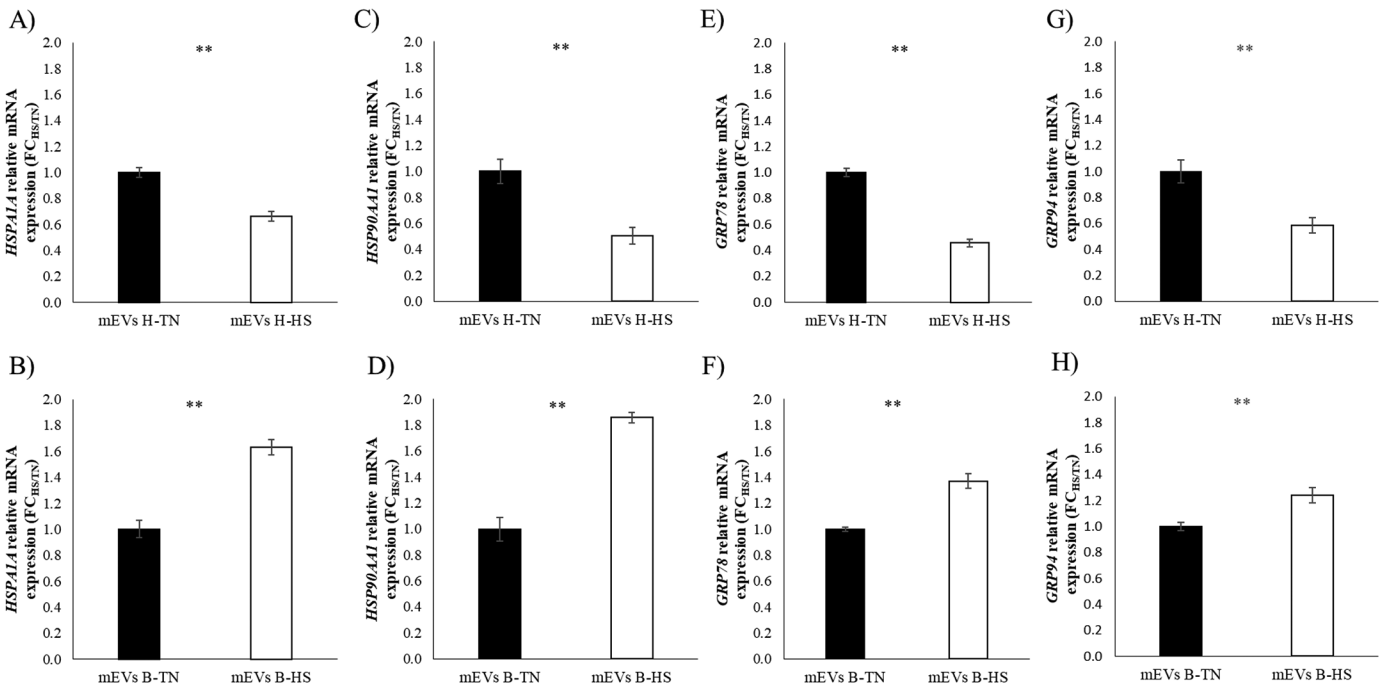

Figure 5. Gene expression of heat shock genes expressed by BME-UV1 was assessed after 24 h of coculture with different types of milk small extracellular vesicles (mEV) in 2 different breeds and environmental conditions. (A) Gene expression of *HSPA1A* in cells treated with mEV from Holstein (H) in thermoneutral (TN) and heat stress (HS) conditions. (B) Gene expression of *HSPA1A* in cells treated with mEV from Brown Swiss (B) in TN and HS conditions. (C) Gene expression of *HSP90AA1* in cells treated with mEVs from Holstein (H) in TN and HS conditions. (D) Gene expression of *HSP90AA1* in cells treated with mEV from Brown Swiss (B) in TN and HS conditions. (E) Gene expression of *GRP78* in cells treated with mEV from H in TN and HS conditions. (F) Gene expression of *GRP78* in cells treated with mEV from B in TN and HS conditions. (G) Gene expression of *GRP94* in cells treated with mEV from H in TN and HS conditions. (H) Gene expression of *GRP94* in cells treated with mEV from Brown Swiss (B) in TN and HS conditions. Data were reported as relative mRNA expression ($FC_{HS/TN}$) ± SE (n = 9). Significant differences between TN and HS treatments of mEVs were reported (**$P < 0.01$).

component. The decreases observed in B cows were lowest compared with H cows. The H cows results confirmed the vulnerability of this breed to HS, as already observed in previous studies (Bernabucci et al., 2014; Amamou et al., 2019; Mylostyvyi et al., 2021; García-Martínez et al., 2022; Xu et al., 2023). Correa-Calderon et al. (2004) observed that during summer in the same barn, the B group showed an RT and RR lower respectively of 0.5°C and 12 breaths/min than the H group. Maggiolino et al. (2020) investigated a specific THI threshold for various Italian B cow milk production characteristics and observed that no THI threshold was linked to a decrease in milk output. The authors also detected that HS negatively influenced protein yield and cheese yield, especially in cows up to the third calving. Only third-parity cows' fat yield was more vulnerable to HS. These findings were confirmed by Maggiolino et al. (2022), who examined the effect of heat waves (**HW**) of 2, 3, 4, and 5 d on some quality traits (FCM, ECM, cheese yield, cheese production at 24 h) in B milk. Data collected in the present study confirms that all traits are negatively affected by HW.

Recent study (Saenz-de-Juano et al., 2023) reported that the molecular cargoes (miRNA and protein abundance) of mEV are not affected by the circadian rhythm, thus the experimental design of the present study included the first milk collection during the morning milking session when the THI was lower than 68 and the cows were in normothermia, while the second milk collection was during the afternoon milking session when the THI was higher than 80 and cows experienced the HS.

The sEV isolated from the milk of H and B cows under HS and TN conditions were studied in vitro to investigate the cellular response of bovine mammary epithelial cells. To date, this study represents the first investigation approaching the response of bovine mammary cells to exposure of mEV isolated from heat-stressed (**HSed**) cows of 2 different breeds. Wang et al. (2021) noted that pretreatments of murine intestinal epithelial cell line (IEC-6) exposed to oxidative stress (H_2O_2) with bovine mEV improved cell viability. Matic and Dia (2022) reported that sEV isolated from bovine milk improved the cellular viability of macrophages under hypoxia conditions. Few studies have investigated the possible messages carried by HSed EV. Gebremedhn et al. (2020) noted that HSed EV carried protective messages in bovine granulosa cells cultivated at 42°C. In contrast, the HSed EV had

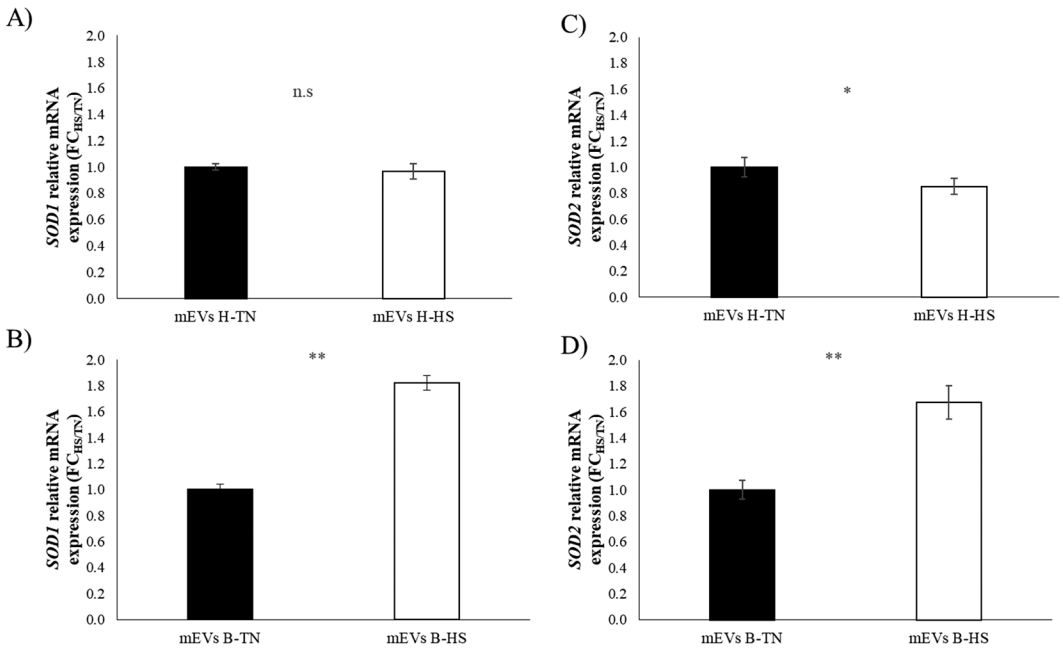

Figure 6. Gene expression of superoxide dismutase family expressed by BME-UV1 assessed after 24 h of coculture with different types of milk small extracellular vesicles (mEV) in 2 different breeds and environmental conditions. (A) Gene expression of *SOD1* in cells treated with mEV form Holstein (H) in thermoneutral (TN) and heat stress (HS) conditions. (B) Gene expression of *SOD1* in cells treated with mEV from Brown Swiss (B) in TN and HS conditions. (C) Gene expression of *SOD2* in cells treated with mEV from Holstein (H) in TN and HS conditions. (D) Gene expression of *SOD2* in cells treated with mEVs from Brown Swiss (B) in TN and HS conditions. Data were reported as relative mRNA expression ($FC_{HS/TN}$) ± SE (n = 9). Significant differences between TN and HS treatments of mEVs were reported (*$P < 0.05$; **$P < 0.01$); n.s. = $P > 0.05$.

deleterious effects on granulosa cells cultured at 37°C and caused a higher percentage of early apoptotic cells and a lower percentage of viable cells. These results are partially consistent with our observation, in which BME-UV1 cells incubated at 37°C and treated with mEV H-HS and B-HS showed an impairment of the cell viability and metabolic activity compared with mEV TN. The mEV HS treatments did not affect the apoptosis activity of BME-UV1 cells. Thus, we may speculate that mammary gland cells or immune cells, under HS, may release EV into the milk delivering signals to modulate the metabolic activity of target cells. The present study showed a reduction of intracellular ROS level in BME-UV1 cells after challenge with mEV H-HS and an increase of ROS level after exposure to mEV B-HS. The opposite response enhanced by mEV HS isolated from H and B breeds may underline that the molecular messages of mEV were different between the 2 breeds. Lacetera et al. (2006) observed a reduction of ROS levels in peripheral blood mononuclear cells (**PBMC**) of Holstein cows incubated at 41, 42, and 43°C compared with the control (39°C). In contrast, in the PBMC of Brown Swiss cows, the ROS level decreased only at a temperature of 42°C. Previous studies suggested that the accumulation of ROS promotes stress resistance by acting as signaling molecules essential to enhance metabolic health and longevity (Poljsak, 2011; Ristow and Schmeisser, 2011). Indeed, different authors (Finkel, 2011; Schieber and Chandel, 2014) reported that ROS are physiological orchestrators of signaling pathways within cells. Therefore, the higher ROS level observed in BME-UV1 cells challenged with mEV B-HS could imply that the molecular message carried by mEV may promote the activation of the defense mechanism against HS in receiving cells. Furthermore, the BME-UV1 cells treated with mEV H-HS showed a downregulation of the expression of genes involved in the HS and antioxidant responses (*HSPA1A*, *HSP90AA1*, *GRP78*, *GRP94*, *SOD2*). On the contrary, BME-UV1 cells cocultured with mEV B-HS promoted upregulation of *HSPA1A*, *HSP90AA1*, *GRP78*, *GRP94*, *SOD1*, and *SOD2*, in agreement with Gebremedhn et al. (2020), who observed an upregulation of *HSP70*, *HSP90*, *GRP78*, *GRP94*, *NRF2*, and *SOD1* in granulosa cells cultured at 37°C treated with HSed EV isolated from the culture medium. The authors argued that granulosa cells activated the adaptive response to HS through HSP gene expression. Some authors (Fang et al., 2021; Hu et al., 2024) reported that *HSPA1A* and *HSP90AA1* are key genes associated with HS tolerance. The upregulation of *HSP* genes obtained in BME-UV1 cells treated with mEV B-HS might indicate that this mEV cargo activated

the cellular cytoprotective mechanism against HS. Mitri et al. (2015) reported that heat shock proteins activate anti-apoptotic mechanisms that prevent the formation of protein aggregates, helping proteins to maintain their structure. This could explain the different tolerance to HS in the H and B breeds both in vitro and in vivo.

This study has some limitations. The experimental design adopted had the main aim to maximize the effect of HS conditions on H and B lactating dairy cattle. Therefore, the first sampling was in the morning milking and the second one was in the afternoon milking after 8 d. Although, some authors (Saenz-de-Juano et al., 2023) reported that miRNA cargo and protein abundance of mEV are not affected by the circadian rhythm, it is known that there are circadian changes involving metabolites, hormones, and cytokines (Teng et al., 2021). Therefore, we may not rule out that some of the changes reported were also related to the circadian rhythm. This hypothesis has to be experimentally verified.

CONCLUSIONS

The molecular response in BME-UV1 cells induced by mEV HS compared with the TN condition is different, and it is also different between the 2 breeds. In particular, the mEV B-HS cell treatment activated protective cellular mechanisms generating an increased ROS level and an overexpression of all genes analyzed. In contrast, the mEV H-HS cell treatment did not stimulate cytoprotective defense as shown by the ROS level reduction and the down-expression of HSP and antioxidant genes. Therefore, this study supported the role played by mEV as molecular orchestrators of physiological conditions delivering different information relating to the thermal stress of the cell and the breed. Further research will profile the molecular cargoes of mEV isolated from different breeds during different thermal conditions to elucidate the possible role of mEV in HS cellular tolerance in cattle.

NOTES

The study was supported by PRIN-2022 grant from Italian Ministry of University and Research (Resiliency to heat stress: A system biology approach, CUP: J53D23010270006). The research was carried out within the framework of the Ministry of University and Research (MUR) initiative "Departments of Excellence" (Law 232/2016) DAFNE Project 2023-27 "Digital, Intelligent, Green and Sustainable" (acronym: D.I.Ver.So). The study was carried out within the Agritech National Research Center and received funding from the European Union Next-GenerationEU (Piano Nazionale di Ripresa e Resilienza [PNRR] – Missione 4 Componente 2, Investimento 1.4 – D.D. 1032 17/06/2022, CN00000022). Supplemental material for this article is available at https://doi.org/10.6084/m9.figshare.27918984. All animal use and procedures for the study were approved by the Ethical Committee for Animal Welfare of Animals employed in scientific research of the Department of Veterinary Medicine of the University of Bari (approval no. 05/2022). This manuscript reflects only the authors' views and opinions; neither the European Union nor the European Commission can be considered responsible for them. The authors have not stated any conflicts of interest.

Nonstandard abbreviations used: aNDFom; = neutral detergent fiber analyses corrected for residual ash content; BME-UV1 = bovine mammary epithelial cell line; CT = threshold cycle; EC = environmental conditions; EV = extracellular vesicles; DCFH-DA = 2′,7′-dichlorodihydrofluorescein diacetate; dFBS = depleted fetal bovine serum; FPCM = fat- and protein-corrected milk; HS = heat stress; HSed = heat stressed; HW = heat waves; mEV = milk EV; MY = milk yield; NTA = nanoparticle tracking analysis; PBMC = peripheral blood mononuclear cells; RFU = relative fluorescence units; ROS = reactive oxygen species; RR = respiration rate; RT = rectal temperature; SEC = size exclusion chromatography; sEV = small EV; THI = temperature-humidity index; TN = thermoneutrality.

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