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Rumen-protected dry grape extract supplementation enhances milk production, behavior traits, and immunometabolism of mid-lactating Fleckvieh cows under naturally occurring heat stress

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ABSTRACT

Heat stress (HS) in dairy cows disrupts homeostasis and thermoregulation, negatively affecting milk production, health status, and metabolism. Dietary supplementation with phytoextracts such as polyphenols may offer an effective dietary strategy by stimulating the immune system, reducing oxidative stress, and ultimately enhancing welfare, metabolic function, and milk performance. This study aimed to evaluate the effects of supplementing mid-lactating Fleckvieh cows with rumen-protected grape extract (Nor-Grape BP-O, Nor-Feed) on milk performance, rectal temperature (RT), behavior, immunometabolism, rumen fermentation, and innate immune response under naturally occurring HS conditions (temperature-humidity index >72). Thirty cows were balanced by days in milk, milk yield, and parity, and randomly assigned to receive either 470 mg/cow per day of Nor-Grape BP-O supplementation (NG-BPO, n = 15) or a control diet without supplementation (CTR, n = 15) and monitored for 35 d. Both groups were cooled daily using forced ventilation and fans. Temperature-humidity index, milk yield, heavy breathing (HB), rumination, and eating time were recorded daily. Milk, blood, and rumen fluid samples were collected at different time points, and RT was measured. The NG-BPO cows exhibited greater milk production, fat-corrected milk, energy-corrected milk, and yields of fat and protein, alongside lower SCC compared with CTR cows. The NG-BPO cows exhibited improved thermoregulatory responses, reflected by significantly lower RT and reduced HB. Supplementation with Nor-Grape BP-O lowered inflammatory status and enhanced innate immune responses, as indicated by higher plasma zinc and myeloperoxidase levels, lower haptoglobin concentrations, and increased phagocytic activity of neutrophils and monocytes compared with CTR cows. These findings were supported by higher levels of proinflammatory cytokines TNF-α and IL-1 β during peak HS periods, compared with CTR cows. In addition, the NG-BPO group had lower circulating platelet and eosinophil counts compared with the CTR group. In conclusion, supplementation with a low dose of rumen-protected dry grape extract—rich in low-molecular-weight polyphenols (oligomeric and monomeric flavanols, anthocyanins)—effectively supported milk production and quality in heat-stressed dairy cows. Moreover, supplementation improved thermotolerance during heat waves and enhanced innate immune functions while reducing inflammatory responses. These findings suggest Nor-Grape BP-O as an effective dietary strategy to mitigate the negative effects of HS by enhancing cow performance and welfare during periods of elevated environmental temperatures.

Key words: heat stress, grape polyphenols, antioxidant, inflammation

INTRODUCTION

Heat stress (**HS**) is one of the major challenges to the global livestock industry, especially for dairy cattle. In fact, compared with other livestock species, dairy cattle are more susceptible to HS, due to genetic selection for high milk yield (**MY**), which leads to increased metabolic

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heat production (Baumgard and Rhoads, 2012; Loor et al., 2023). In dairy cattle, the thermoneutral zone—the range in which they do not need to expend additional energy to maintain thermal balance and remain in a state of comfort—ranges from 5 to 25°C (Becker et al., 2020). When thermal comfort is compromised and animals are exposed to temperatures outside this range, physiological and behavioral homeostasis mechanisms are triggered to help maintain thermal balance. A temperature-humidity index (**THI**) above 72 typically indicates a HS condition in dairy cattle (Heinicke et al., 2018). However, it has been shown that cows producing more than 35 kg/d of milk start to exhibit HS at a THI of 68 (Collier et al., 2012).

However, although animals can adapt to adverse environmental conditions, prolonged exposure and extreme temperatures can impair cow welfare. The initial response to HS is increase in water intake, sweating, respiratory rate, and heart rate, along with a decrease in feed intake (Horowitz, 2002). All these challenges and adaptations in the long term compromise production, reproduction, and animal health, with economic losses for the farm. Indeed, Garner et al. (2017) estimated a reduction in milk production ranging from 17% to 53%, partly linked to decreased DMI, which accounts for only 35% to 50% of the HS-induced decrease in milk synthesis (Rhoads et al., 2009; Wheelock et al., 2010). The reduction in DMI and the alteration of energy metabolism also triggers a reduction in milk quality, especially a decrease in milk fat yield and, occasionally, also in protein and lactose yields (Florio et al., 2022). Moreover, a recent study reported that economic losses due to HS in Italian dairy farms are estimated at $23.57 to $43.98 per farmer per day, mainly from reduced milk quality rather than yield (Moore et al., 2023). However, economic losses reflect not only impaired production performance but also the physiological consequences of HS.

Health-related problems in heat-stressed dairy cows are mainly related to the resulting oxidative stress (Chauhan et al., 2014) and immune suppression. During HS, oxidative stress occurs when the production of reactive oxygen species exceeds the capacities of antioxidant defense mechanisms, impairing immune function and increasing the susceptibility of cows to disease (Alberghina et al., 2024).

The impairment of the animal's immune status is caused, in part, by reductions in neutrophil phagocytosis and lymphocyte proliferation (Tejaswi et al., 2020), which play important roles in defending the body against infection and disease. Consequently, their decline increases the animal's vulnerability to infectious agents. Cows exposed to high environment THI had reduced blood plasma concentration of inflammatory cytokines and immunoglobulins (Safa et al., 2019).

The most adopted method to minimize the negative influence of HS is the use of evaporative cooling, fans, and water sprinklers (Mader et al., 2007). However, these heat abatement strategies have been demonstrated to be insufficient to fully mitigate the negative effects on dairy cows' productivity during the summer months (Baumgard and Rhoads, 2012; Collier et al., 2019) and require infrastructure investments as well as energy and water costs, affecting the sustainability and profitability of the dairy industry (von Keyserlingk et al., 2013). The dietary concentrations of vitamins, minerals, antioxidants, and other specific nutrients can improve productivity of dairy cows under HS (Sun et al., 2019). The inclusion of polyphenols in dairy cow diets may offer a valuable strategy to reduce oxidative stress associated with HS, due to their well-known antioxidant and anti-inflammatory properties (Zhang and Tsao, 2016). Grape polyphenols, known for their antioxidant properties, have been shown to enhance the humoral immune response and antioxidant activity in young cattle (Engler et al., 2022).

Based on the well-established antioxidant and anti-inflammatory properties of grape extract, this study aimed to evaluate the effects of supplementing heat-stressed dairy cows with rumen-protected dry grape extract (Nor-Grape BP-O, Nor-Feed) on milk production and quality, behavior, immunometabolic response, and oxidative stress. We hypothesize that supplementation with rumen-protected dry grape extract (Nor-Grape BP-O) will improve thermotolerance, enhance immune and antioxidant responses, and sustain milk production and quality in dairy cows exposed to a natural condition of HS and during summer heat waves.

MATERIALS AND METHODS

Animal Management and Treatment

The experimental procedures for the trial were approved by the University of Messina Animal Care Committee (number 06/2024 *bis*) in accordance with ARRIVE guidelines and regulations. The trial was conducted at the Fattoria Demetra Srl Soc. Agricola commercial farm in Calabria (South Italy) during the peak of summer (July–August), under natural HS conditions typical of the summer period. A total of 30 Fleckvieh (dual-purpose Simmental) primiparous and multiparous (parity 2–6) mid-lactation (170 ± 61 DIM) cows were randomly assigned to dietary groups fed for 5 consecutive weeks, according to MY, parity, and DIM: (1) standard milking cow diet (control, **CTR**, n = 15), or (2) standard milking cow diet supplemented with 470 mg/d of Nor-Grape BP-O (a rumen-protected dry grape extract rich in water-soluble polyphenols; **NG-BPO**, n = 15).

The supplement consisted of a dry grape extract (*Vitis vinifera*, EU 2b485), rumen-protected with an organic plant matrix to avoid alteration of the phenolic profile and activity in the rumen. It contained 60% total polyphenols, 45% oligomeric procyanidins, and 0.6% anthocyanins. The NG-BPO supplement was mixed with corn meal (100 g) and top-dressed individually onto TMR once a day in the morning at 0700 h, providing a daily dietary supply of 282 mg of bioactive polyphenols per cow. The CTR group received, instead, an equivalent amount of corn meal without supplementation as a placebo. During supplementation, all cows enrolled in the trial were head-locked with 1 empty space between each animal, as the pen where they were housed had a feed bunk with 65 head-locks. Each cow remained individually head-locked until the top-dress was completely consumed and was monitored throughout the entire process. Approximately 1 week before the start of the experimental trial, the time required for a cow to fully consume the top-dress (consisting solely of corn meal) was simulated to assess the feasibility and reliability of this supplementation strategy. As a result, this approach was adopted during the trial, because each cow took ~15 s to consume the entire top-dress. All cows were housed in the same pen and fed ad libitum once a day as TMR with a common diet, summarized in Table 1. Although all cows were housed in a single pen, supplementation was managed at the individual level to allow accurate monitoring of the 2 treatments. Cows were milked 2 times daily at 0500 and 1700 h in an 8-herringbone milking parlor. All animals of both groups were cooled all days by forced ventilation in the free-walking resting area (straw-bedded), which automatically turned on when THI exceeded 64. The feeding area was equipped with forced fans and a big-drop soaker system along the cow feed-rack (CMP Animal Welfare, Calvisano, Italy). Even in this area, fans automatically turned on when THI exceeded 64, whereas soakers automatically turned on when THI exceeded 68 and were activated for 1 min at 5-min intervals from 0800 to 2000 h each day throughout the trial. The rotation intensity of fans was regulated according to the THI detected by appropriate probes inside the barns (CMP Animal Welfare). Ambient temperature and relative humidity to calculate the THI were recorded every 15 min by 3 probes installed in the feeding and resting areas and in the milking parlor (CMP Animal Welfare). The THI was calculated based on the equation reported by (Ouellet et al., 2021): $THI = (1.8 \times T + 32) - [(0.55 - 0.0055 \times RH) \times (1.8 \times T - 26)]$, where T = ambient temperature (°C) and RH = relative humidity (%). The daily mean, maximum, and minimum THI registered throughout the trial are reported in the Figure 1.

After ~25 d of treatment, the THI registered was above 70 until 33 d of treatment, identifying that period of increase in THI as a heat wave. As defined by Hahn (1999), the registration of a prolonged period of excessively hot weather is defined as a heat wave: at least 3 d with a mean THI greater than or equal to 70.

Table 1. Ingredients and chemical composition of the formulated and analyzed experimental diet

Item	% of DM
Ingredient	
Grass hay	25.4
Alfalfa hay, second cut or later	18.1
Corn grain, ground, dry	21.6
Commercial lactating concentrate mix[1]	20.0
Steam-flaked corn	9.4
Molasses	2.2
Mineral and vitamin mix[2]	2.1
Hydrogenated fats	1.2
Nutrient composition,[3] % of DM	
CP	16.83
Starch	23.95
Sugars	5.33
Ether extract	5.40
NDF	38.75
ADF	16.76
ADL	4.30
Ash	6.90
NEL,[4] Mcal/kg of DM	1.65

[1]Contained solvent-extracted flour of toasted soybean, solvent-extracted flour of sunflower, dried stillage corn, whole cottonseed, wheat bran, alfalfa flour, middling wheat, calcium carbonate, carob flour, fatty acid salts of palm oil, sodium chloride, phosphate dicalcium, sodium bicarbonate. Nutrient composition, as-fed basis: 12.50% DM, 33.00% CP, 6.00% ether extract, 10.50% crude fiber, 10.5% ash, and 0.65% Na.

[2]As-fed basis: 11.50% Ca, 5.20% P, 12.00% Na, 2.20% Mg, 400,000 IU/kg rumen-protected vitamin A, 100,000 IU/kg vitamin D_3, 3,000 mg/kg vitamin E, 8,000 mg/kg niacinamide, 8,000 mg/kg choline, 40 mg/kg biotin, 800 mg/kg Cu, 800 mg/kg Mn, 1,500 mg/kg chelate Zn, 3,000 mg/kg Zn oxidase, 60 mg/kg I, 20 mg/kg Se, 25,000 mg rumen-protected methionine (Mepron, Evonik), *Saccharomyces cerevisiae* 20 Mcfu/kg.

[3]Values represent an average of TMR nutrient analysis from samples collected each day throughout the trial (35 d). Diet DM averaged 60.82% ± 2.05%.

[4]Calculated using NRC (2001).

Animal Behavior Recordings and Rectal Temperature Measurement

All cows were equipped with SenseHub sensor collars (MSD Animal Health) that monitored and transmitted data to the installed SenseHub dairy system antenna during the entire period of the trial. The SenseHub sensor collars recorded cow activities and transmitted them every 20 min. Sensor collar data were then preprocessed by the manufacturer and provided by MSD Animal Health. The data set included the parameters "Rumination," "Eating," and "Overheat" (herein stated as "heavy breathing"; defined as minutes per hour when the respiration rate measured by the monitoring neck tags is equal to or above 80 acts per minute) and were stated for every hour as minutes per hour (min/h). Finally, processed sensor collar data were organized as minutes per hour (min/h)

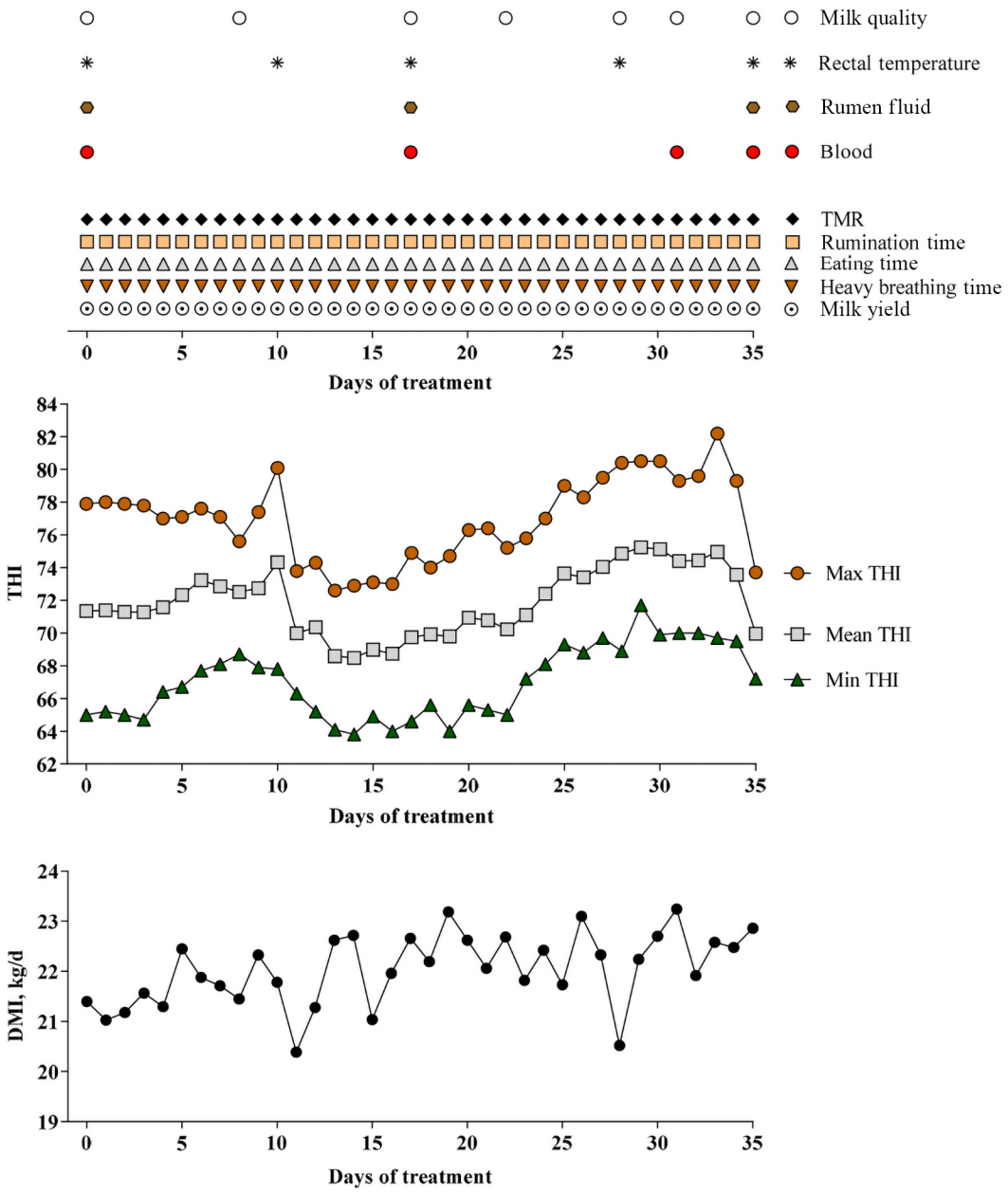

Figure 1. Experimental design with sampling time points, THI trends, and daily DMI averages of all lactating cows enrolled in the trial under the same environmental conditions, throughout the experimental entire period. Mid-lactating Fleckvieh cows during summer were divided into 2 groups and fed for 35 d either a standard diet (CTR) or a diet supplemented with rumen-protected dry grape extract at 470 mg/head per day (NG-BPO). The supplementation of rumen-protected dry grape extract was mixed with corn meal and top-dressed onto TMR for each cow once a day in the morning. The CTR group received, instead, an equivalent amount of corn meal without supplementation. Max = maximum; min = minimum.

for all the parameters considered, and then a daily time budget was also calculated for all days during the trial (35 d).

Rectal temperature (**RT**) was recorded at 0, 10, 17, 28, and 35 d of treatment immediately after the morning milking (0800 h), at 1400 h, and immediately after the evening milking (2000 h). The RT was recorded using a clinical digital thermometer (TFA Dostmann Thermometer VET 112) inserted to a depth of ~10 cm, and measurements were always performed by the same operator, to minimize variability.

Milk Yield, Sample Collection, and Analysis

Milk yield was automatically recorded at each milking (twice daily, summed for total MY/d) during the 35-d

trial period. Milk samples were collected at 0, 9, 17, 22, 28, 31, and 35 d. Samples were taken from 2 consecutive milkings: the first collection occurred in the evening, and the second was collected on the following morning. This strategy was implemented to reduce bias from the variations in TMR meals provided to cows throughout the day, as both milk samples were taken from cows fed TMR over a single day rather than over 2 d. Milk samples were analyzed separately (evening and morning) for fat, protein, lactose, casein, urea, total milk solids, nonfat milk solids, citric acid, and free fatty acids by mid-infrared spectroscopy using a Milkoscan FT2 (Foss Electric A/S, 3400 Hillerød, Denmark) and for SCC using a Fossomatic FC (Foss Electric) at the DALILAB laboratory of the University of Messina Department of Veterinary Sciences (Messina, Italy). After analysis, the overall milk quality parameters were calculated by averaging the individual samples, weighted according to the volume of milk produced in each respective milking. This approach ensured that the composite values accurately reflected the milk characteristics, considering the contribution of each sampling based on production volume.

Blood Sampling for Plasma and Hematology Analysis

Blood samples were collected by jugular venipuncture into 10-mL heparinized and K_3 EDTA tubes (BD Vacutainer, Becton, Dickinson and Co., Franklin Lakes, NJ) before the morning feeding. Blood samples with heparinized tubes for plasma analyses were collected at 0, 17, 31, and 35 d of treatment, whereas blood samples with K_3 EDTA tubes for hematology analysis were collected at 0, 17, and 35 d of treatment.

Heparinized tubes were immediately placed into an ice-water bath, centrifuged within 1 hour of collection, and subsequently processed as described by Lopreiato et al. (2021) for the assessment of a wide profile of blood-based biomarkers in plasma. Briefly, commercial kits were used to measure glucose, total cholesterol, urea, calcium, inorganic phosphorus, magnesium, total protein, albumin, total bilirubin, and creatinine (Instrumentation Laboratory SpA, Milan, Italy), nonesterified (free) fatty acids (**NEFA**), alkaline phosphatase (ALP), and zinc (Wako, Chemicals GmbH, Neuss, Germany), and BHB (Ranbut Kit, Randox Laboratories Ltd., Crumlin, UK). Electrolytes (Na^+, K^+, and Cl^-) were detected by the potentiometer method (ion-selective electrode connected to an ILab 650 analyzer [Instrumentation Laboratory Company, Lexington, MA]). Kinetic analysis was adopted to determine the activity of enzymes: aspartate aminotransferase (AST, EC 2.6.1.1) and γ-glutamyl transferase (GGT, U/L, EC 2.3.2.2), using commercial kits (Instrumentation Laboratory SpA, Milan, Italy). The total globulin fraction was determined by subtracting albumin from total protein. Haptoglobin and ceruloplasmin concentrations were measured using methods proposed by Bertoni et al. (2008) adapted to ILab 650 conditions. Plasma paraoxonase (PON, EC 3.1.8.1) activity was assessed by adapting the method of Ferré et al. (2002) to the ILab 650. Oxidative stress intensity was evaluated by measuring total reactive oxygen metabolites (**ROM**) as described by Bionaz et al. (2007); activity of myeloperoxidase (**MPO**) was determined using a colorimetric method described by Bradley et al. (1982). This assay is based on the ability of MPO in plasma to catalyze the oxidation of O-dianisidine dihydrochloride in the presence of hydrogen peroxide (H_2O_2), resulting in the formation of a colored product measurable at 460 nm. Ferric-reducing antioxidant power (**FRAP**) was measured using the colorimetric method of Benzie and Strain (1996), as well as advanced oxidation protein products (**AOPP**) as described by Hanasand et al. (2012). Thiol group levels (**SHp**) were measured using a Diacron kit (Diacron International, Grosseto, Italy), which assesses total plasma thiols, including both low-molecular-weight free thiols and protein-bound thiols (mainly albumin thiol groups). However, the method does not differentiate between the 2 fractions. Plasma from blood samples at 17 and 31 d of treatment was also assessed for IL1β, IL-6, IL-10, IFN-γ, and TNF-α. These biomarkers were measured using commercial ELISA kits following the manufacturer's recommendations. Briefly, a 100-µL plasma sample was used for the analysis with sandwich ELISA. The microplate was precoated with an antibody specific to cytokines. The samples were added to the microplate wells and combined with the specific antibody. Then, a biotinylated detection antibody specific for each cytokine and avidin-horseradish peroxidase conjugate were added successively to each microplate well and incubated. Free components were washed away. Substrate solution was added to each well. The enzyme-substrate reaction was determined by optical density (**OD**) and measured spectrophotometrically at a wavelength of 450 nm in a plate reader (Victor-X3, Perkin Elmer). The concentration of each cytokine was calculated by comparing the OD of the samples against the standard curve.

Blood samples collected into K_3 EDTA were stored in an insulated container at room temperature and analyzed within 3 h for a complete blood count by an automatic clinical hematology device (Sysmex XN-1000, Sysmex Corp., Kobe, Japan). Parameters considered in the current study were red blood cells, white blood cells, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin (**MCH**), MCH concentration, red cell volume distribution width, platelet count, mean platelet volume, plateletcrit, lymphocytes, monocytes, neutrophils, eosinophils, and basophils.

Phagocytosis Capacity of Neutrophils and Monocytes

At 35 d of treatment, in addition to the blood sample for plasma and hematology, a specific blood sample was collected in a 10-mL heparinized tube to assess the phagocytic capacity of neutrophil and monocyte cells according to the method described by Hulbert et al. (2011). Briefly, 2 whole blood aliquots of 200 µL were transferred into 2 tubes: 1 for the control and 1 with the addition of 80 µL of fluorescently labeled *Escherichia coli*. The tubes were incubated at 38.5°C for 10 min and then cooled in an iced bath for 10 min. The erythrocytes were hypotonically lysed, adding 800 µL of iced Milli-Q PF water (Merck KGaA), and then quickly adding 200 µL of 5× PBS to avoid excessive damage of cells. The tubes were centrifuged at 990 × *g* for 5 min at 4°C, and the supernatant was discarded. The lysing process was repeated 3 times. Then, the cells were washed using 2 mL of PBS 1× and centrifuged at 990 × *g* for 5 min at 4°C. Cells were resuspended with 200 µL of PBS 1× and analyzed with flow cytometry (Attune NxT Flow Cytometer, Thermo Fisher). Data were analyzed according to Scatà et al. (2024) using flow-cytometer analysis software (Accuri C6 Plus Software, BD Biosciences) to obtain the percentages of phagocytic cells.

Rumen Samples and Gas Chromatography for VFA Analysis

At 0, 17, and 35 d of treatment, rumen fluid was collected 4 h after the morning TMR was provided, using a specially designed ruminal probe for cattle (Ruminator, profs-products.com), as outlined by Wallace et al. (2019). To minimize salivary contamination, the first 400 mL of fluid collected was discarded, and the following 500 mL was retained. The pH of all samples was immediately measured with a pH meter (GLP 21, Crison Instruments SA). The pH meter was calibrated every time before use with standard buffer solutions (pH 4.0, 7.0, and 10.0) to ensure accuracy (XS Basic, XS Instruments). Subsequently, the samples were gently mixed, and 4 aliquots (1.5 mL each) were pipetted into 2-mL tubes for storage at −20°C until VFA analysis.

Rumen fluid samples were prepared by centrifugation at 15,000 × *g* for 10 min to obtain a clear supernatant for analysis. The concentrations of VFA in the rumen fluid were assessed using a gas chromatograph (model 7820A, Agilent Technologies). Additionally, urea, as well as D- and L-lactic acid levels in the rumen fluid, were measured following the methods described by Minuti et al. (2014). Individual VFA concentrations were reported as concentration and molar proportions of the total VFA concentration. Urea levels were estimated using a urea nitrogen kit (Instrumentation Laboratory Werfen) with a clinical autoanalyzer (ILAB-650); after that, NH_3-N was obtained dividing the urea value by 2 (approximation). Both D- and L-lactic acids were measured using a D-/L-lactate assay kit (Megazyme International Ltd.), adhering to the manufacturer's instructions.

Statistical Analysis

Data were analyzed with SAS software (release 9.3, SAS Institute Inc.). Normal distribution of the residuals was assessed using the UNIVARIATE procedure in SAS. Parameters that were not normally distributed were subjected to logarithmic transformation (i.e., haptoglobin, AST, GGT, bilirubin, NEFA, and BHB). After analysis, residuals were plotted to assess model assumptions of normality and homoscedasticity. Milk, plasma, hematology, SenseHub sensors collar (rumination, eating, and heavy breathing, **HB**), and RT data were analyzed as repeated measurements with the GLIMMIX procedure of SAS. The statistical models included the fixed effects of treatment (CTR and NG-BPO), time (days of treatment), and their interaction, with individual cows included as random effect. Phagocytic activities of neutrophils and monocytes were analyzed with the GLM procedure of SAS, where the model included only the fixed effect of treatment (CTR and NG-BPO), as blood samples for this assessment were collected only at 35 d of treatment. For repeated measurements, 4 structures of covariance (compound symmetry, autoregressive order, Toeplitz, or spatial power) were tested, and the one with the lowest Akaike information criterion was retained in the model. Variables non-normally distributed were log-transformed, and, once the output data were carried out from the GLIMMIX procedure, they were back-transformed. All means were compared using the PDIFF statement of SAS, and Tukey-Kramer's post hoc adjustment was applied. Statistical significance was declared at $P \leq 0.05$ and tendencies at $0.10 \geq P > 0.05$.

Finally, 2-phase segmented regressions were performed on the least squares means of MY and HB data retrieved from the mixed models when daily THI was added to the model. Segmented regressions were created using the NLIN procedure to detect THI breakpoints at which variables begin to significantly rise or decline in CTR and NG-BPO cows. Differences among correlations coefficients between treatments were tested with a Fisher-type Z-transformation, and significance was set at $P \leq 0.05$.

RESULTS

Milk Production and Quality

Results of milk performance are reported in Table 2. Dietary supplementation of Nor-Grape BP-O affected MY, which was overall greater in the NG-BPO group compared with the CTR group (29.3 vs. 27.4 ± 0.25 kg/d for NG-BPO and CTR, respectively; treatment effect, **Trt**; $P < 0.01$). Figure 2A reports the trend of MY during the 35 d of treatment. In addition, in Figure 2B, a threshold for the THI was identified for both groups. The threshold identification is based on significant changes in MY, when exceeding a specific THI value. In particular, the CTR cows had a THI breakpoint for MY of THI 72.70, where MY began decreasing at a rate of −1.65 kg/d for every increase of 1 THI unit above the threshold. In NG-BPO cows, the THI breakpoint identified was 72.83, above which cows' MY decreased at a rate of −1.42 kg/d for every 1 unit of THI above the threshold. The Fisher-type Z-transformation test revealed that the correlations between THI and MY in the 2-phase segmented regression were similar between the CTR group (adjusted [**adj.**] r = 0.51; adj. $R^2 = 0.26$) and the NG-BPO group (adj. r = 0.49; adj. $R^2 = 0.24$), with a *P*-value of 0.46 (Z-value = 0.11).

Among milk performance results (Table 2), the NG-BPO group showed overall greater FCM (NG-BPO: 30.14, vs. CTR: 28.43 ± 1.58 kg/d; Trt; $P < 0.01$) and ECM (NG-BPO: 30.36, vs. CTR: 28.81 ± 1.82 kg/d; Trt; $P < 0.01$), as well as greater fat (NG-BPO: 1.09, vs. CTR: 1.01 ± 0.06 kg; Trt; $P = 0.01$) and protein (NG-BPO: 0.97, vs. CTR: 0.91 ± 0.06 kg; Trt; $P = 0.02$) yields, compared with the CTR group. Additionally, NG-BPO cows showed overall lower SCC (NG-BPO: 87.85, vs. CTR: 112.36 ± 27.03 × 10^3 cells/mL; Trt; $P = 0.05$), compared with the CTR group.

Rectal Temperature and Behavior Response

Table 3 reports results of RT, HB, rumination, and eating time.

Among the measurement of HB, a significant interaction effect (Trt × Time; $P < 0.01$) was observed, where NG-BPO cows showed a lower HB compared with the CTR group, especially after 25 d of treatment and in correspondence with the occurrence of the heat wave (Figure 2C). As shown in Figure 2D, the THI breakpoint detected for CTR cows in relation to HB was 71.88, whereby HB began rising at a rate of 26.06 min/d for every unit increase of THI above the threshold. A different trend was observed for NG-BPO cows, where the THI breakpoint was lower (70.80) compared with CTR cows. However, despite the lower threshold, the increase registered in HB beyond the breakpoint was lower than in CTR cows, with an increase of only 4.78 min/d for every unit increase in THI. The Fisher-type Z-transformation test revealed that the correlation between THI and HB in the 2-phase segmented regression was significantly stronger in the CTR group (adj. r = 0.94; adj. $R^2 = 0.88$) compared with the NG-BPO group

Table 2. Milk yield and composition of mid-lactating Fleckvieh cows fed either a standard diet (CTR, n = 15) or a diet supplemented with rumen-protected dry grape extract at 470 mg/head per day (NG-BPO, n = 15) for 35 d during a natural HS exposure in summer

	Treatment[1]			*P*-value[2]		
Item	CTR	NG-BPO	SEM[3]	Trt	Time	Trt × Time
Milk yield, kg/d	27.38	29.22	1.63	<0.01	<0.01	0.88
Fat-corrected milk 4%, kg/d	28.43	30.14	1.58	<0.01	<0.01	0.85
Energy-corrected milk, kg/d	28.81	30.36	1.82	<0.01	<0.01	0.91
Fat, %	3.89	3.93	0.19	0.78	0.06	0.92
Fat, kg	1.01	1.09	0.06	0.01	<0.01	0.49
Protein, %	3.46	3.43	0.08	0.65	<0.01	0.86
Protein, kg	0.91	0.97	0.06	0.02	<0.01	0.92
Lactose, %	4.58	4.53	0.07	0.51	<0.01	0.55
Casein, %	2.72	2.69	0.06	0.61	<0.01	0.72
Urea, mg/dL	30.47	29.77	1.51	0.59	<0.01	0.66
Total milk solids, %	13.01	12.94	0.22	0.72	<0.01	0.84
Nonfat milk solids, %	9.15	9.05	0.11	0.29	<0.01	0.57
Citric acid, %	0.14	0.13	0.11	0.49	<0.01	0.87
Somatic cell count, ×10^3 cells/mL	112.36	87.85	27.03	0.05	0.15	0.49

[1]Cows were fed a control TMR diet (CTR, n = 15) or a control diet supplemented with rumen-protected dry grape extract at 470 mg/head/day (NG-BPO, n = 15). Throughout the trial, the average mean THI was 74, the average maximum THI was 77, and the average minimum THI was 67.

[2]Trt = overall effect of dietary supplementation (CTR vs. NG-BPO); Time = overall effect of days relative to start of supplementation; Trt × Time = interaction of treatment and days relative to start of supplementation.

[3]Greatest SEM.

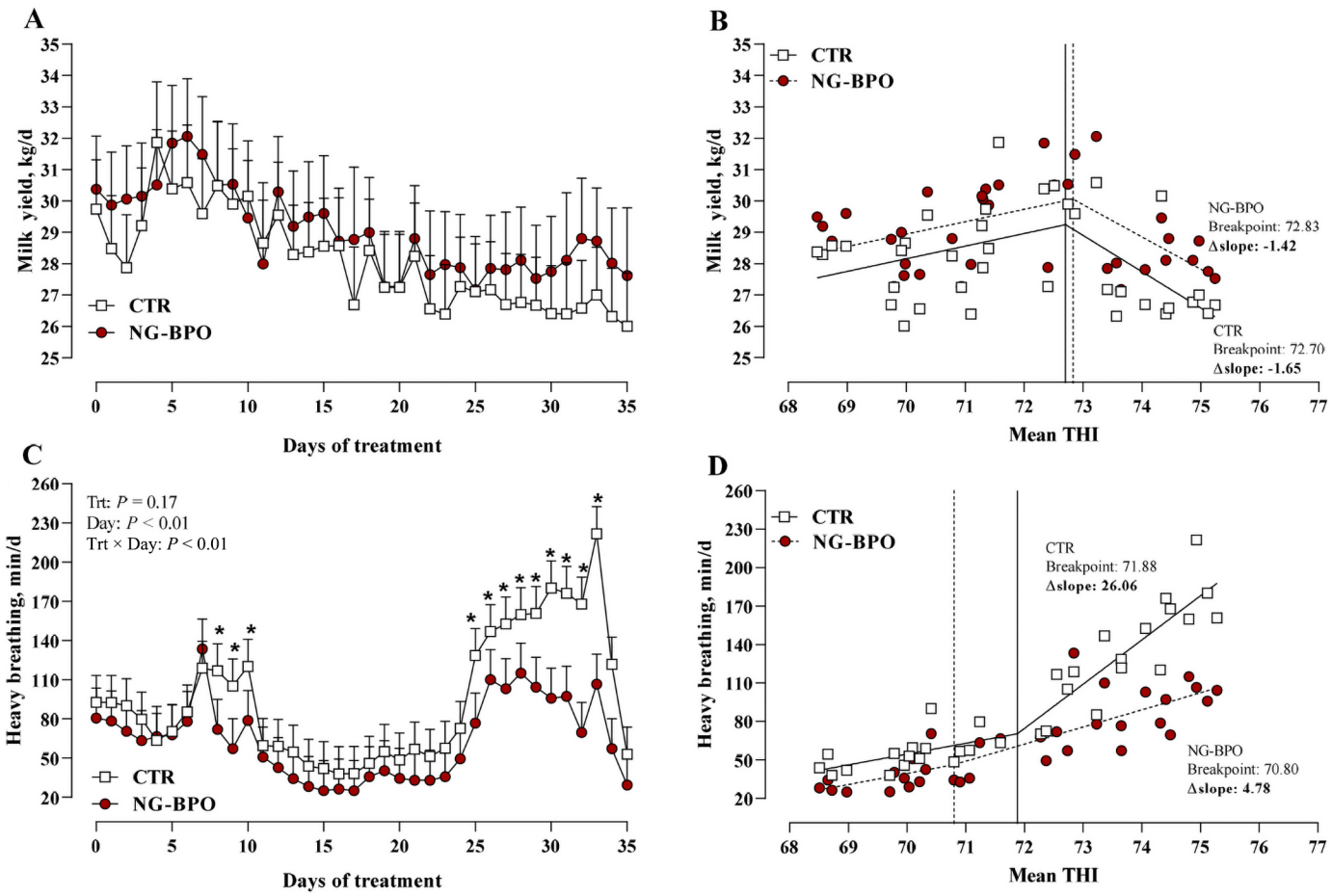

Figure 2. Trend milk yield (A) with segmented regressions relative to daily mean THI (B) and trend of heavy breathing (C) with segmented regressions relative to daily mean THI (C) of mid-lactating Fleckvieh cows fed either a standard diet (CTR, n = 15) or a diet supplemented with rumen-protected dry grape extract at 470 mg/head per day (NG-BPO, n = 15) for 35 d during a natural HS exposure in summer. Error bars represent SE (A and C). Differences between groups within each time point ($P \leq 0.05$) are denoted with an asterisk (C). Vertical lines indicate breakpoint at which milk yield (B) and heavy breathing (D) changed significantly and abruptly in NG-BPO cows (dashed lines) and in CTR cows (solid lines). Δ slope represents the change in slope between the slope of data before breakpoint (b_1) and the slope of data after breakpoint (b_2).

Table 3. Diurnal rectal temperature, heavy breathing, rumination time, and eating duration of mid-lactating Fleckvieh cows fed either a standard diet (CTR) or a diet supplemented with rumen-protected dry grape extract (NG-BPO) for 35 d during a natural HS exposure in summer

	Treatment[1]			*P*-value[2]		
Parameter	CTR	NG-BPO	SEM[3]	Trt	Time	Trt × Time
Rectal temperature, °C						
0800 h	38.75	38.60	0.12	0.07	0.01	0.05
1400 h	38.73	38.79	0.17	0.54	<0.01	0.87
2000 h	38.72	38.76	0.19	0.61	<0.01	0.80
Heavy breathing, min/d	100.89	68.65	15.47	0.17	<0.01	<0.01
Rumination, min/d	527.67	514.22	21.08	0.49	<0.01	0.17
Eating, min/d	220.99	254.75	15.66	0.05	<0.01	0.87

[1]Cows fed a control TMR diet (CTR, n = 15) or a control diet supplemented with rumen-protected dry grape extract at 470 mg/head per day (NG-BPO, n = 15). Throughout the trial, the average mean THI was 74, the average maximum THI was 77, and the average minimum THI was 67.

[2]Trt = overall effect of dietary supplementation (CTR vs. NG-BPO); Time = overall effect of days relative to start of supplementation; Trt × Time = interaction of treatment and days relative to start of supplementation.

[3]Greatest SEM.

(adj. r = 0.84; adj. R^2 = 0.70), with a *P*-value of 0.02 (Z-value = 2.07).

Overall, the RT parameter showed a tendency at 0800 h in the NG-BPO cows compared with the CTR cows (Trt: *P* = 0.07), with also a significant interaction effect (Trt × Time; *P* = 0.05). However, no significant differences between groups were detected at 1400 and 2000 h (*P* > 0.05). During the experimental period, the RT at 0800 h was significantly lower at 17 (NG-BPO: 38.70, vs. CTR: 38.82 ± 0.06°C), 28 (NG-BPO: 38.63, vs. CTR: 38.9 ± 0.07°C), and 35 (NG-BPO: 38.54, vs. CTR: 38.7 ± 0.07°C) days of treatment (*P* < 0.05) in the NG-BPO cows compared with CTR cows (Figure 3).

Overall, the eating time was greater (Trt; *P* = 0.05) in NG-BPO compared with CTR cows, but no significant differences between groups were observed for rumination time (*P* > 0.05).

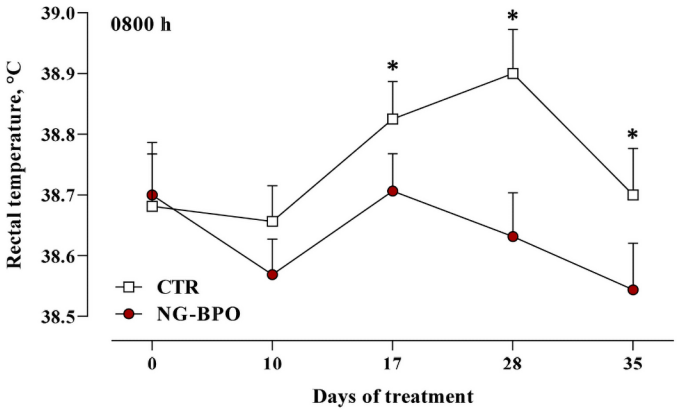

Figure 3. Rectal temperature (°C) of mid-lactating Fleckvieh cows fed either a standard diet (CTR) or a diet supplemented with rumen-protected dry grape extract at 470 mg/head per day (NG-BPO) for 35 d during a natural HS exposure in summer. Differences between groups within each time point ($P \leq 0.05$) are denoted with an asterisk. Error bars represent SE.

Blood Biomarkers

The response in plasma biomarkers related to energy metabolism, liver functionality, oxidative stress and antioxidant status, minerals, and inflammatory response is summarized in Table 4. No statistical differences between groups were observed for plasma biomarkers related to energy metabolism (*P* > 0.05). However, for parameters of inflammatory response, an interaction effect (Trt × Time; *P* = 0.05) was observed for haptoglobin, with NG-BPO cows showing lower levels after 31 d of treatment (NG-BPO: 0.15, vs. CTR: 0.31 ± 0.06 g/L; *P* = 0.04; Figure 4A). Conversely, MPO concentration was greater, showing a significant interaction effect (Trt × Time; *P* = 0.04), where NG-BPO cows exhibited greater levels of MPO after 35 d of treatment (NG-BPO: 384.3, vs. CTR: 355.4 ± 12.9 U/L; *P* = 0.02; Figure 4B) compared with CTR cows. Also, Zn levels were greater in NG-BPO cows, with both a treatment (Trt; *P* = 0.04) and an interaction effect (Trt × Time; *P* = 0.05), with a greater blood concentration of Zn at 17 (NG-BPO: 13.95, vs. CTR: 10.91 ± 0.85 µmol/L; *P* = 0.01) and 31 d (NG-BPO: 12.57, vs. CTR: 10.59 ± 0.85 µmol/L; *P* = 0.05), compared with CTR cows (Figure 4C).

Among parameters of liver functionality, statistical differences between groups were observed. Specifically, total protein concentration was overall greater in NG-BPO cows compared with CTR cows (Trt; *P* = 0.02). In addition, a treatment effect was observed for globulin concentration, being overall greater in the NG-BPO group compared with the CTR group (Trt; *P* = 0.01), and an interaction effect was also observed (Trt × Time; *P* = 0.05), with NG-BPO cows showing greater globulin levels at 17 (NG-BPO: 45.26, vs. CTR: 42.29 ± 0.86 g/L; *P* = 0.01), 31 (NG-BPO: 45.58, vs. CTR: 42.66 ± 0.86 g/L; *P* = 0.02), and 35 d of treatment (NG-BPO: 44.03, vs. CTR: 40.20 ± 0.86 g/L; *P* = 0.002) compared with CTR cows (Figure 4D). The albumin-to-globulin ratio was overall lower in the NG-BPO group than CTR group (Trt; *P* = 0.02). An interaction effect (Trt × Time; *P* = 0.04; Figure 4E) was also observed, with the ratio lower at 31 (NG-BPO: 0.81, vs. CTR: 0.86 ± 0.01 g/L; *P* = 0.02) and 35 d (NG-BPO: 0.82, vs. CTR: 0.89 ± 0.01 g/L; *P* = 0.001) in the NG-BPO cows compared with CTR cows. In addition, the bilirubin concentration was overall lower (Trt; *P* = 0.04) in NG-BPO cows compared with CTR cows.

Plasma Cytokines

Table 5 summarizes the differences between NG-BPO and CTR groups in blood cytokines. As shown in Figure 5A, TNF-α blood concentration was significantly greater (Trt × Time; *P* = 0.04) in NG-BPO cows after 31 d of treatment and during the occurrence of the heat wave (NG-BPO: 10.91, vs. CTR: 9.26 ± 0.88 pg/mL; *P* = 0.05) compared with CTR cows. Additionally, IL-1β levels were, overall, higher in NG-BPO cows compared with CTR cows (Trt; *P* = 0.04), and no interaction effect was observed (Trt × Time; *P* > 0.05; Figure 5B). No Trt or Trt × Time effect was obtained for IL-6, IL-10, or IFN-γ (*P* > 0.05; Table 5).

Hematology Response

The hematology response of heat-stressed dairy cows supplemented or not with Nor-Grape BPO is reported in Table 6. The platelet count was overall lower in NG-BPO cows (Trt; *P* = 0.03) compared with CTR cows, with an interaction effect as well (Trt × Time; *P* = 0.03), being

Table 4. Blood biomarkers of energy metabolism, liver function, inflammatory response, oxidative stress and antioxidant status, and minerals in mid-lactating Fleckvieh cows fed either a standard diet (CTR) or a diet supplemented with rumen-protected dry grape extract (NG-BPO) for 35 d during a natural HS exposure in summer

	Treatment[1]			*P*-value[2]		
Biomarker[3]	CTR	NG-BPO	SEM[4]	Trt	Time	Trt × Time
Energy metabolism						
Glucose, mmol/L	3.88	3.90	0.07	0.68	0.01	0.68
BHB, mmol/L	0.62	0.65	0.06	0.51	0.01	0.38
NEFA, mmol/L	0.11	0.12	0.02	0.69	0.39	0.20
Creatinine, µmol/L	101.17	102.52	2.22	0.53	0.01	0.74
Urea, mmol/L	7.45	7.32	0.33	0.67	<0.01	0.77
Liver functionality						
Total protein, g/L	77.45	80.23	1.31	0.02	0.01	0.15
Albumin, g/L	35.56	36.13	0.50	0.18	0.06	0.55
PON, U/mL	78.83	80.77	2.08	0.10	0.07	0.88
Cholesterol, mmol/L	5.45	5.82	0.29	0.26	0.02	0.94
Globulin, g/L	41.75	44.22	0.92	0.01	<0.01	0.05
Albumin-to-globulin ratio	0.87	0.83	0.16	0.02	0.07	0.04
AST, U/L	98.37	101.70	5.67	0.51	0.11	0.75
GGT, U/L	27.17	28.45	0.81	0.11	0.03	0.25
Bilirubin, µmol/L	1.58	1.32	0.22	0.04	<0.01	0.47
ALP, U/L	98.88	109.52	6.45	0.17	0.10	0.70
Inflammatory response						
Ceruloplasmin, µmol/L	1.89	1.88	0.08	0.96	<0.01	0.89
Haptoglobin, g/L	0.19	0.13	0.06	0.10	0.11	0.05
Zn, µmol/L	11.20	12.53	0.87	0.04	0.52	0.05
MPO, U/mL	378.76	383.52	13.95	0.70	0.35	0.04
Oxidative stress and antioxidant status						
ROMt, mg of H_2O_2/0.1 L	12.63	12.53	0.60	0.87	<0.01	0.87
FRAP, µmol/L	150.78	153.07	4.40	0.55	0.45	0.57
SHp, µmol/L	303.24	306.08	9.57	0.75	0.15	0.42
AOPP, µmol/L	29.65	31.44	1.42	0.15	<0.01	0.81
Minerals						
Ca, mmol/L	2.61	2.62	0.04	0.61	0.13	0.56
P, mmol/L	2.26	2.25	0.12	0.96	0.18	0.78
Mg, mmol/L	0.93	0.92	0.02	0.64	<0.01	0.64
Na, mmol/L	145.20	145.41	1.30	0.80	0.64	0.18
K, mmol/L	4.27	4.16	0.13	0.42	<0.01	0.11
Cl, mmol/L	102.46	102.56	1.17	0.88	0.14	0.25

[1]Cows fed a control TMR diet (CTR, n = 15) or a control diet supplemented with rumen-protected dry grape extract at 470 mg/head per day (NG-BPO, n = 15). Throughout the trial, the average mean THI was 74, the average maximum THI was 77, and the average minimum THI was 67.

[2]Trt = overall effect of dietary supplementation (CTR vs. NG-BPO); Time = overall effect of days relative to start of supplementation; Trt × Time = interaction of treatment and days relative to start of supplementation.

[3]NEFA = free fatty acids; PON = paraoxonase; AST = aspartate aminotransferase; GGT = gamma-glutamyl transferase; ALP = alkaline phosphatase; MPO = myeloperoxidase; ROMt = total reactive oxygen metabolites; FRAP = ferric-reducing ability of plasma; SHp = thiol groups; AOPP = advanced oxidation protein products.

[4]Greatest SEM.

lower at 17 d of treatment (NG-BPO: 405.63, vs. CTR: 524.21 ± 27.10 cells × 10^6/mL; $P = 0.001$). Overall, the eosinophil percentage was significantly lower in the NG-BPO group compared with the CTR group (Trt; $P = 0.03$), with an additional interaction effect (Trt × Time; $P = 0.05$), showing lower levels after 35 d of treatment (NG-BPO: 3.52, vs. CTR: 6.51 ± 0.64%; $P = 0.001$). Additionally, the eosinophil count was also decreased in NG-BPO cows compared with CTR cows, with both treatment effect (Trt; $P = 0.05$) and interaction effect (Trt × Time; $P = 0.05$), where, compared with CTR cows, NG-BPO cows showed fewer circulating eosinophils at 35 d of treatment (NG-BPO: 0.30, vs. CTR: 0.52 ± 0.06 cells × 10^6/mL; $P = 0.05$).

Phagocytosis

Results of neutrophil and monocyte phagocytosis activity are shown in Figure 6. In detail, monocyte phagocytosis activity was greater in NG-BPO compared with CTR cows (NG-BPO: 46.81, vs. CTR: 41.67 ± 2.58%; Trt: $P = 0.05$; Figure 6A), as was neutrophil phagocytosis activity (NG-BPO: 39.32, vs. CTR: 32.08 ± 2.32%; Trt: $P = 0.03$; Figure 6B).

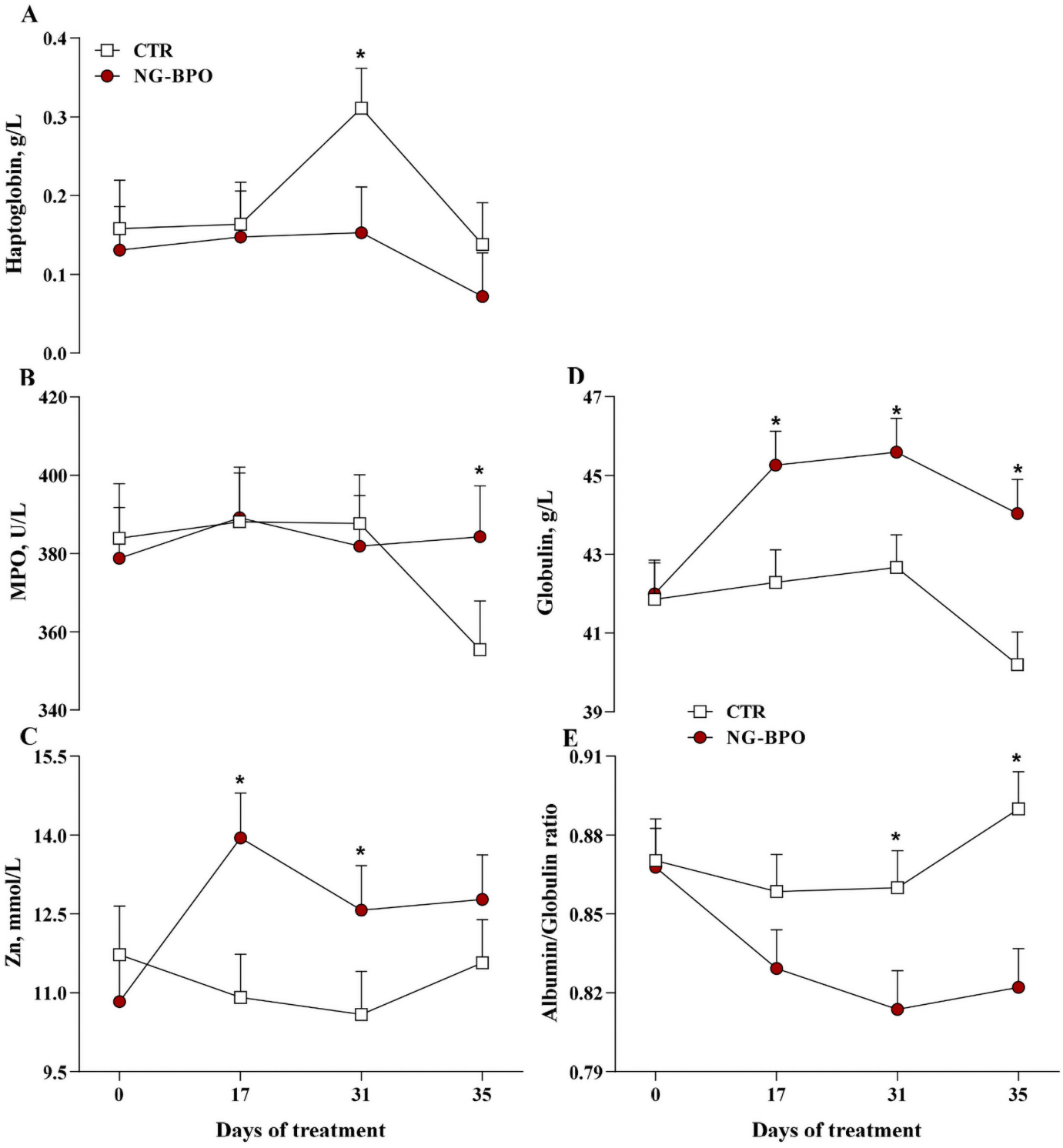

Figure 4. Plasma haptoglobin (A), myeloperoxidase (MPO; B), zinc (Zn; C), globulin (D), and albumin-to-globulin ratio (E) of mid-lactating Fleckvieh cows fed either a standard diet (CTR) or a diet supplemented with rumen-protected dry grape extract at 470 mg/head per day (NG-BPO) for 35 d during a natural HS exposure in summer. Differences between groups within each time point ($P \leq 0.05$) are denoted with an asterisk. Error bars represent SE.

Table 5. Plasma cytokines in mid-lactating Fleckvieh cows fed either a standard diet (CTR) or diet supplemented with rumen-protected dry grape extract (NG-BPO) for 35 d during a natural HS exposure in summer

	Treatment[1]			*P*-value[2]		
Biomarker, pg/mL	CTR	NG-BPO	SEM[3]	Trt	Time	Trt × Time
IL-1β	22.70	26.21	2.83	0.04	0.28	0.57
IL-6	6.58	6.77	0.62	0.68	0.01	0.68
IL-10	61.37	39.17	34.06	0.38	0.44	0.63
IFN-γ	22.70	21.97	1.70	0.57	0.60	0.90
TNF-α	9.27	9.88	1.21	0.49	0.23	0.04

[1]Cows fed a control TMR diet (CTR, n = 15) or a control diet supplemented with rumen-protected dry grape extract at 470 mg/head per day (NG-BPO, n = 15). Throughout the trial, the average mean THI was 74, the average maximum THI was 77, and the average minimum THI was 67.

[2]Trt = overall effect of dietary supplementation (CTR vs. NG-BPO); Time = overall effect of days relative to the start of the supplementation; Trt × Time = interaction of treatment and days relative to start of supplementation.

[3]Greatest SEM.

Rumen Fluid Data

The effects of dietary supplementation of Nor-Grape BP-O on rumen fluid pH, lactates, ammonia, and VFA are shown in Table 7. A tendency for an interaction effect (Trt × Time; $P = 0.09$) was observed for L-lactate, which was lower in NG-BPO cows compared with CTR cows at 17 d of treatment (NG-BPO: 82.71, vs. CTR: 98.51 ± 7.19 mg/dL; $P = 0.05$). Differences between groups were also observed for rumen pH, which tended to be higher in NG-BPO cows at 17 d of treatment (NG-BPO: 6.49, vs. CTR: 6.40 ± 0.06; $P = 0.09$), but on the contrary, when THI markedly decreased after the heat wave at 35 d (from ~74 on average to ~70), NG-BPO cows showed a lower rumen pH than CTR cows (NG-BPO: 6.37, vs. CTR: 6.48 ± 0.06; $P = 0.09$).

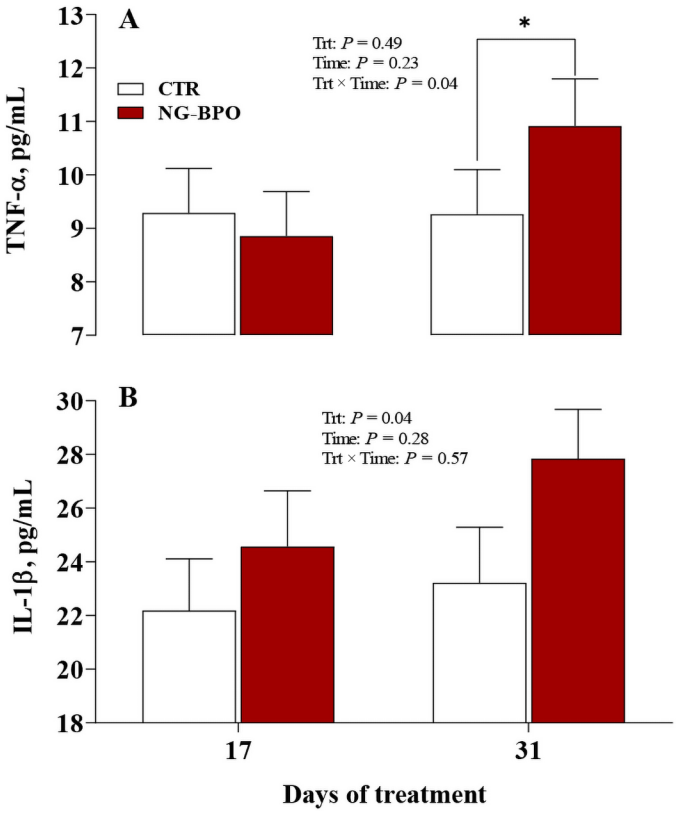

Figure 5. Plasma tumor necrosis factor-α (TNF-α; A) and interleukin-1 β (IL-1β; B) of mid-lactating Fleckvieh cows fed either a standard diet (CTR) or a diet supplemented with rumen-protected dry grape extract at 470 mg/head per day (NG-BPO) for 35 d during a natural HS exposure in summer. Differences between groups within each time point ($P \leq 0.05$) are denoted with an asterisk. Error bars represent SE.

DISCUSSION

Heat stress is a challenge to the dairy industry, due to the increase in global surface temperature in recent years, especially during the summer months. In these months, the risk of increased mortality is greater, due to the occurrence of heat waves and the increase of THI levels (Vitali et al., 2015).

The use of dietary supplements during HS has the main objective of reducing body temperature, to maintain or increase milk production and quality, and to prevent oxidative stress and immunity disfunctions in animals. In this context, previous studies have shown that plant polyphenols exert antioxidant properties, which can not only help to improve oxidative status, but also support productivity, inflammatory response, and immunomodulation (Shakoor et al., 2021; Piao et al., 2023).

Among the different sources of dietary polyphenols (olive, tea, cocoa, coffee; Cardona et al., 2013), this study investigated whether the supplementation of rumen-protected dry grape extract in the diet of heat-stressed Fleckvieh cows could help mitigate the negative effects of HS during summer. Moreover, being rumen-protected, the polyphenols' effects could be more efficient, bypassing rumen degradability and being absorbed by the small intestine; in fact, previous studies have demonstrated

Table 6. Hematology response of mid-lactating Fleckvieh cows fed either a standard diet (CTR) or diet supplemented with rumen-protected dry grape extract (NG-BPO) for 35 d during a natural HS exposure in summer

	Treatment[1]			*P*-value[2]		
Item	CTR	NG-BPO	SEM[3]	Trt	Time	Trt × Time
Red blood cells, $\times 10^6$/μL	6.12	6.13	0.18	0.95	<0.01	0.59
White blood cells, $\times 10^6$/mL	8.18	8.41	0.48	0.50	0.31	0.74
Hemoglobin, g/dL	9.61	9.60	0.20	0.94	<0.01	0.89
Hematocrit, %	30.05	30.03	0.71	0.97	<0.01	0.89
Mean corpuscular volume, fL	49.52	49.14	0.85	0.55	0.01	0.56
Mean corpuscular hemoglobin (MCH), Pg	15.64	15.99	0.51	0.47	0.11	0.52
MCH concentration, g/dL	32.01	31.97	0.24	0.82	<0.01	0.92
Red cell volume distribution width, %	19.20	18.91	0.57	0.47	0.07	0.57
Platelets, $\times 10^6$/mL	402.42	354.09	35.20	0.03	<0.01	0.03
Mean platelet volume, fL	7.97	8.06	0.15	0.32	<0.01	0.52
Plateletcrit, %	0.29	0.25	0.04	0.13	0.05	0.47
Lymphocytes, %	55.11	56.42	3.47	0.57	<0.01	0.26
Monocytes, %	1.87	1.74	0.26	0.41	<0.01	0.42
Neutrophils, %	37.02	37.43	3.40	0.85	<0.01	0.61
Eosinophils, %	5.21	3.83	1.04	0.03	0.24	0.05
Basophils, %	1.03	0.98	0.22	0.67	0.17	0.93
Lymphocyte count, $\times 10^6$/mL	4.43	4.79	0.37	0.17	0.06	0.28
Monocyte count, $\times 10^6$/mL	0.15	0.14	0.02	0.44	<0.01	0.50
Neutrophil count, $\times 10^6$/mL	3.60	3.18	1.17	0.55	0.99	0.32
Eosinophil count, $\times 10^6$/mL	0.41	0.31	0.08	0.05	0.11	0.05
Basophil count, $\times 10^6$/mL	0.09	0.09	0.02	0.62	<0.01	0.55

[1]Cows fed a control TMR diet (CTR, n = 15) or a control diet supplemented with rumen-protected dry grape extract at 470 mg/head per day (NG-BPO, n = 15). Throughout the trial, the average mean THI was 74, the average maximum THI was 77, and the average minimum THI was 67.

[2]Trt = overall effect of dietary supplementation (CTR vs. NG-BPO); Time = overall effect of days relative to the start of supplementation; Trt × Time = interaction of treatment and days relative to start of supplementation.

[3]Greatest SEM.

that grape polyphenol's antioxidant activity is degraded in ruminal fluid (Chedea et al., 2016). Furthermore, previous studies have demonstrated how the use of rumen-protected grape extract needs a potential protection to preserve their activity (Engler et al., 2022).

Supplementing Nor-Grape BP-O in heat-stressed dairy cows not only prevented the decline in milk production but overall enhanced it. Moreover, evaluating the relationship between the mean THI and MY indicated that the NG-BPO group had a higher THI breakpoint compared with the CTR group. The data also indicate that, above the THI breakpoint within each group, the CTR group experienced a decline of 1.65 kg/d in milk production, whereas the NG-BPO group showed a smaller decrease of 1.42 kg/d. This suggests that the NG-BPO group was more adaptive to HS and was able to maintain a higher level of MY compared with the CTR group under elevated THI conditions. Previous studies have investigated the effect of supplementing dried grape on milk production, and Gessner et al. (2015) reported results similar to those of this study, whereas other reported no significant differences (Nielsen and Hansen, 2004; Ianni et al., 2019; Pauletto et al., 2020). In this context, it is interesting to determine whether the differences in polyphenol amounts may have influenced milk production and related parameters. The different results reported in previous studies could be related to variation in the form of grape polyphenols used (e.g., grape pomace vs. purified extract), polyphenol concentrations, treatment duration, and basal diet composition. In this study, cows received 282 mg of rumen-protected polyphenols per head per day for 35 d, whereas in the study by Gessner et al. (2015), the amount of polyphenols per cow per day was ~6,240 mg for dry cows and 11,440 mg for lactating cows for 90 d. However, in the experiment by Nielsen and Hansen (2004), cows were fed with 4.5 g of grape pomace per day, but the amount of polyphenol content was not specified, as was also the case in the study by Pauletto et al. (2020). In the study by Ianni et al. (2019), each cow received 2 kg of dried grape pomace, corresponding to ~32,540 mg of polyphenols per cow per day, showing no significant differences in milk production and quality compared with cows without supplementation. Meanwhile, Nudda et al. (2019) reported an increase in MY in lactating ewes fed with grape marc, where the amount of polyphenols per head was ~1,480 mg/d. The authors suggested that the polyphenols may have interacted with proteins, forming complexes and thus reducing protein solubility and ruminal degradation, and potentially increasing the amount of protein digested in the small intestine. Therefore, the better digestion and nutrient availability of proteins may lead to greater milk production (Patra and Saxena,

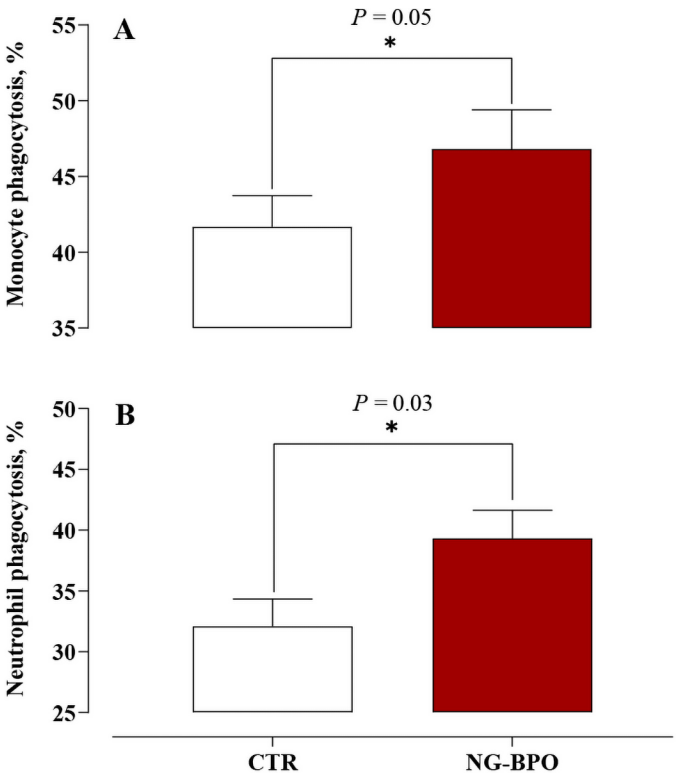

Figure 6. Monocyte (A) and neutrophil (B) phagocytosis activity against *Escherichia coli* at 35 d of treatment of mid-lactating Fleckvieh cows fed either a standard diet (CTR) or a diet supplemented with rumen-protected dry grape extract at 470 mg/head per day (NG-BPO) for 35 d during a natural HS exposure in summer. Differences between groups within each time point ($P \leq 0.05$) are denoted with an asterisk. Error bars represent SE.

2011). This result is also supported by the greater ECM and FCM showed by the NG-BPO group compared with the CTR group, and by the greater contents of milk yield fat and protein. Moreover, the greater milk production and FCM content may be also supported by the lower RT showed in the NG-BPO cows, as a signal that NG-BPO cows exhibited lower energy expenditures for body cooling at the expense of milk production, as previously reported by Kim et al. (2022).

Somatic cell count usually increases during HS, due to the reduced immunity of animals (Negri et al., 2021). Previously, Nudda et al. (2019) reported no effect on SCC with a supplementation of 100 g of grape per ewe per day, providing 1,480 mg of polyphenols per ewe per day. Furthermore, Huang et al. (2019) also did not observe differences in SCC after supplementing up to 80 mg of a grape seed extract per kilogram of body weight per day; this extract contained 95% procyanidins, equivalent to supplementing 53,200 mg of nonprotected polyphenols per cow per day for a 700-kg cow. However, in the present study, cows supplemented with NG-BPO (equivalent to 282 mg of protected polyphenols per cow per day) exhibited a decrease in SCC compared with the CTR group, suggesting a better immunostimulatory response and a healthier mammary gland even with lower polyphenol amounts, that could be attributed to the presence of rumen-protected polyphenols. Thus, these results suggest that this is the optimal dose of this supplement to ensure maintenance of milk production and quality during heat load.

In the present study, the supplementation of rumen-protected dry grape extract significantly decreased both RT and HB of dairy cows, suggesting that feeding Nor-Grape BP-O helped alleviate body heat production, thus enabling the animals to dissipate heat more effectively. Increased respiration rate is one of the primary cooling mechanism in dairy cows for heat load, reaching up to 200 breaths per minute during a HS condition (Mader et al., 2006). In contrast, a lower respiration rate is related to more efficient heat dissipation, which could be potentially linked to genetic or physiological traits (Collier et al., 2019). The CTR group exhibited significantly greater HB compared with the NG-BPO group, especially during the last week of the trial and in correspondence with the heat wave. This affirmed that, whereas the CTR group experienced a condition of HS, Nor-Grape BP-O supplementation had the potential to alleviate respiratory effort caused by HS. This finding is further supported by the more consistent and tightly fitted relationship between HB and THI in the CTR group ($R^2 = 0.88$) compared with NG-BPO cows ($R^2 = 0.70$), and the significantly stronger correlation coefficient in CTR cows between the 2 variables ($Z = 2.07$; $P = 0.02$). Moreover, the rapid escalation in HB in the CTR group after the THI breakpoint, with an increase of 26.06 min/d compared with 4.78 min/d in the NG-BPO group, for each unit of THI above the threshold, suggests a better ability to cope with HS, with a more moderate increase of HB in NG-BPO group with rising THI.

Rectal temperatures were different between the groups, especially during the morning. Indeed, the NG-BPO group exhibited lower RT at 17, 28, and 35 d of treatment. This result showed that the NG-BPO group was more able to dissipate heat, maintaining a lower body temperature, although these cows had to support greater milk production compared with the CTR cows. During HS, the occurrence of oxidative stress involves high production of free radicals and oxygen-reactive species, damaging cells and tissues and also consequently affecting immune function (Bernabucci et al., 2002). In this context, polyphenol supplementation as feed additives has been shown to exert anti-inflammatory effects during HS (Gessner et al., 2017). Although in this study no significant differences between groups were observed in biomarkers of oxidative stress (total ROM, SHp, FRAP, and AOPP), it is noteworthy that polyphenols also have

Table 7. Rumen fluid pH, lactates, ammonia, and VFAs of mid-lactating Fleckvieh cows fed either a standard diet (CTR) or diet supplemented with rumen-protected dry grape extract (NG-BPO) for 35 d during a natural HS exposure in summer

	Treatment[1]			*P*-value[2]		
Item	CTR	NG-BPO	SEM[3]	Trt	Time	Trt × Time
pH	6.45	6.42	0.07	0.66	0.95	0.09
NH_3-N, mg/dL	22.34	20.26	1.58	0.10	0.07	0.73
D-Lactate, mg/dL	88.96	86.20	8.99	0.63	0.07	0.67
L-Lactate, mg/dL	85.67	80.65	9.18	0.34	0.01	0.09
Total VFA, mmol/L	91.20	91.82	3.68	0.78	0.04	0.55
Individual VFA, mmol/L						
Acetate	58.97	58.98	2.06	0.99	0.09	0.68
Propionate	18.86	19.39	1.68	0.66	0.01	0.53
Butyrate	10.62	10.43	0.53	0.52	0.96	0.54
Isobutyrate	0.79	0.78	0.04	0.68	0.13	0.75
Valerate	0.88	0.89	0.04	0.67	0.01	0.49
Isovalerate	0.89	0.94	0.06	0.21	0.09	0.71
Hexanoate	0.26	0.27	0.03	0.40	0.27	0.57
Heptanoate	0.02	0.03	0.00	0.32	0.58	0.69
Individual VFA, mol/100 mol						
Acetate	64.85	64.54	0.83	0.64	<0.01	0.46
Propionate	20.39	20.82	1.13	0.60	<0.01	0.53
Butyrate	11.62	11.42	0.32	0.28	0.04	0.70
Isobutyrate	0.89	0.87	0.06	0.55	0.02	0.54
Valerate	0.95	0.97	0.24	0.24	<0.01	0.89
Isovalerate	0.96	1.04	0.13	0.46	0.14	0.71
Hexanoate	0.28	0.30	0.03	0.40	0.08	0.55
Heptanoate	0.03	0.03	0.03	0.27	0.32	0.72

[1]Cows fed a control TMR diet (CTR, n = 15) or a control diet supplemented with rumen-protected dry grape extract at 470 mg/head per day (NG-BPO, n = 15). Throughout the trial, the average mean THI was 74, the average maximum THI was 77, and the average minimum THI was 67.

[2]Trt = overall effect of dietary supplementation (CTR vs. NG-BPO); Time = overall effect of d 0, 17, and 35 relative to start of supplementation; Trt × Time = interaction of treatment and days relative to start of supplementation.

[3]Greatest SEM.

wider effects on the organism. A recent study on pullets (Aberkane et al., 2025) has demonstrated that polyphenols can exert pleiotropic effects on animal physiology, extending beyond their well-known antioxidant capacity. Indeed, in that study, supplementation of Nor-Grape showed significant effects on early skeletal development and modulation of metabolomic pathways of pullets, improving bone mineralization and strength and reducing the prevalence of bone defects. Therefore, the improved thermoregulation and immune response observed in the current trial may result from polyphenol-driven metabolic and cellular adaptations, rather than (or in addition to) direct oxidative stress mitigation.

Herein, haptoglobin was lower in NG-BPO cows compared with CTR cows after 31 d of treatment. Haptoglobin is the main positive acute-phase protein in cows, occurring during inflammatory status and oxidative stress (Bertoni and Trevisi, 2013), and higher levels were previously associated with HS conditions (Maggiolino et al., 2025). From the results of this study, the availability of polyphenols in Nor-Grape BP-O may have supported and regulated inflammation of dairy cows during HS conditions, resulting in contrasting blood haptoglobin levels. Although the decrease in haptoglobin levels may reflect a reduction in systemic inflammation, the increase in MPO could suggest a localized activation of innate immunity.

Myeloperoxidase (MPO) is an enzyme released by neutrophils during bacterial killing, contributing to antimicrobial defense (Bradley et al., 1982). In our study, MPO concentrations remained relatively stable throughout the trial in both NG-BPO and CTR groups, except at 35 d, when stable activity was observed in the NG-BPO group, whereas a marked decrease occurred in CTR cows. This pattern may reflect the enhanced neutrophil activation in the NG-BPO group at the end of the trial, which coincided with increased THI and the occurrence of a heat wave. This response might indicate enhanced immune activity in NG-BPO cows, whereas CTR cows appeared less reactive under the same environmental stress. Thus, the decrease in haptoglobin and the increase in MPO may indicate differential modulation of the immune system. In fact, the presence of polyphenols can modulate immune response in both innate and adaptive systems, having both stimulatory and inhibitory effects (Hachimura et al., 2018).

The better inflammatory response is also confirmed by the greater levels of Zn concentration in blood of NG-BPO cows. Zinc is an important mineral, which is sequestered into hepatocytes during the acute-phase response (Rink and Kirchner, 2000); thus its concentration is lower during severe inflammatory conditions. Previous studies (Amato et al., 2024) have reported lower blood haptoglobin and greater Zn concentrations in cows after supplementation of olive cake rich in polyphenols (~560.32 mg of polyphenols per cow per day for 30 d), with an effect of improving the liver function and at the same time inducing a less pronounced inflammatory response. Polyphenols are known for their antioxidant, antiradical, and radical-scavenging activities, and previous studies have also indicated that they can act as zinc ionophores (Dabbagh-Bazarbachi et al., 2014), facilitating Zn transport across cell membranes and potentially increasing its bioavailability. Thus, we can assume that the greater Zn concentration in NG-BPO cows, which is related to its better bioavailability, may reflect both reduced systemic inflammation and a more efficient zinc-mediated immune function.

Several studies have previously reported decreased plasma glucose in cows under HS, mainly arising from low DMI, and higher levels of plasma insulin (Wheelock et al., 2010). The alteration of feed intake during HS and consequent negative energy balance can also alter carbohydrate, lipid, and protein metabolism (Calamari et al., 2011). Although this study found no differences in biomarkers related to energy metabolism, differences were observed for biomarkers of liver function. Indeed, total protein concentration was greater in NG-BPO cows. Blood proteins are mainly represented by albumin and globulin, which are synthetized by the liver and, in smaller measure, by the immune system (Eckersall, 2008). Serum globulins are indicators of cows' immune response, and they include immunoglobulins (Bionaz et al., 2007). Indeed, immunoglobulins (Ig) are a functionally and structurally differentiated class of globulins that act as antibodies. Most Ig belong to the class of IgG, with an important role in protecting the body from infection and disease, being involved in the adaptive immune response. Although in this study the concentration of IgG was not measured, we can speculate that the greater plasma globulin concentration in the NG-BPO group during the different time points of the treatment (17, 31, and 35 d), could be supportive of greater Ig production and thus a better adaptive immune response of NG-BPO cows. In support of this speculation, the study of Engler et al. (2022) demonstrated that 670 mg of Nor-Grape BP-O per cow per day increases the humoral response due to the presence of polyphenols (Shakoor et al., 2021), and this evidence has been found not only in cattle but also in other species supplemented with grape extracts (Hao et al., 2015; Ao and Kim, 2020).

As a reflection of the greater globulin concentration of the NG-BPO group, the albumin-to-globulin ratio (**A:G**) was lower at 31 and 35 d of treatment compared with the CTR group. Although the decrease of A:G is associated with inflammatory status in dairy cows (Cattaneo et al., 2021) during critical productive periods such as at dry-off or during the transition period, our speculation is that in this case (during HS) A:G could not be related to an inflammatory response, as its value was within the physiological range (Bionaz et al., 2007), but, probably, the lower ratio in NG-BPO cows could be associated with lower levels of dehydration (lower albumin levels) as well as a stimulation of humoral response (greater globulins).

It is noteworthy that during HS the liver function is altered, caused by nutritional and metabolic alterations (Kim et al., 2022). The impairment of liver function is a consequence in bilirubin concentration in blood serum, which is increased in heat-stressed animals (Cincovic et al., 2011). Thus, as confirmed by our results, the lower bilirubin concentration in NG-BPO cows could be related to better hepatic function, as a consequence of better excretory capacity of the liver. The hypothesis of better liver functionality is supported by results of Gessner et al. (2015), who observed, after supplementing dairy cows with a polyphenol-rich extract derived from grapes, a significant reduction in the mRNA expression of genes associated with inflammation and endoplasmic reticulum stress in the liver. Interestingly, this improvement, in the present study, was obtained with only 282 mg of rumen-protected polyphenols per cow per day, compared with 11,440 mg unprotected polyphenols per cow per day in the Gessner et al. (2015) study (22 kg of DMI per cow per day, containing 1% of grape product at 52 mg of polyphenols per gram).

The alteration of the production and distribution of cytokines during HS is mainly related to the triggering of the hypothalamic-pituitary-adrenal axis and increasing peripheral glucocorticoid levels (Kim et al., 2022). In fact, by the consequent stimulation of blood cortisol concentration, cytokine production is inhibited (Kim et al., 2022).

Previous studies have documented reduction of immune response in heat-stressed dairy cows, associated with lower production of TNF-α (Lacetera et al., 2005; Caroprese et al., 2009). At 17 d of treatment, TNF-α levels were initially lower in the NG-BPO group, but after 31 d of supplementation they increased. As reported by Lendez et al. (2021), the diminishment of this cytokine during HS makes the animals less physiologically prepared to resist high temperatures, having a less ef-

ficient immune response and, hence, making them more susceptible to opportunistic infections. This finding was supported also by Safa et al. (2019), who observed higher production of TNF-α in cooled cows compared with heat-stressed cows during the postpartum period, defining this increase as fundamental activation of a moderate and controlled inflammation. Thus, the lower TNF-α in the CTR group could be related to a signal of dysregulation of inflammatory responses (Safa et al., 2019) in this group, which was well supported by its higher production in the NG-BPO group. Indeed, TNF-α has an important role in stimulating the phagocytosis activity of neutrophils and in mobilizing macrophages and neutrophils to the site of infection (Whiteside, 2007).

Interleukin-1β (IL-1β) is a proinflammatory cytokine, which, through stimulation of diapedeses and chemoattraction of neutrophils and monocytes, is also important in promoting phagocytosis (Peker and Musal, 2022). In an in vitro HS-induced study, isolated PMN reduced the production and release of IL-1β (Lopreiato et al., 2020), an effect corroborated by the downregulation of immune and oxidative stress-related genes, thus compromising the overall immune response and cells' ability to effectively counteract pathogenic challenges. Therefore, the greater concentration of this proinflammatory cytokine in NG-BPO cows shows a normal degree of endogenous inflammation, which is necessary to positively regulate both innate and adaptive mechanisms, to ensure nutrient requirements to support the mammary gland and milk synthesis (Bradford et al., 2015).

Because NG-BPO cows exhibited less panting and lower RT, they likely experienced less physiological pressure, which probably contributed to maintaining lower circulating blood cortisol. Although in this study blood cortisol was not measured, the greater IL-1β and TNF-α could be indicative of reduced cortisol secretion, as cortisol can inhibit cytokine expression under HS conditions (Bagath et al., 2019) and due to its immunosuppressive role. Therefore, the different trend in NG-BPO cows might point to better immune function during naturally occurring HS in those cows as well as a more efficient resolution of the systemic inflammatory response, as supported by the lower haptoglobin levels, especially during the heat wave. This may potentially support cytokine functionality, thus reducing the negative immunosuppressive effects typically observed under HS conditions.

Taken together, this response may indicate enhancement of innate and adaptive immune functions, helping NG-BPO cows to maintain a more effective immune response. Indeed, this is confirmed by the better phagocytosis activity in the NG-BPO group compared with the CTR group, as shown by greater phagocytosis activity of both neutrophils and monocytes. Moreover, the immunomodulation observed with dry grape extract rich in polyphenols during HS could be attributed to the polyphenol-mediated modulation of intracellular signaling pathways, such as NF-κB and MAPK. This results in the upregulation of proinflammatory cytokines (e.g., TNF-α, IL-1β) and enhanced responsiveness of innate immune cells, including neutrophils and monocytes.

The variation of season and, thus, environmental temperature also influences hematological parameters. During HS, hematological alteration may be related to disease (Park et al., 2021) or to the increased consumption of water usually detected with rising temperature, which results in hemodilution (Casella et al., 2013). Although in this study we did not observe significant differences between groups on these blood parameters, platelets, eosinophils proportion, and eosinophils count were lower in NG-BPO cows. Unexpectedly, in our study and in contrast with these previous studies, supplementation of rumen-protected grape extract was associated with a decrease in blood platelets. However, studies in mice have shown that stress conditions enhance the increase of platelet activity, as, after stress exposure, sympathetic stimulation of norepinephrine and epinephrine induces platelet activation (Chen et al., 2016). The result of lower blood eosinophils in the NG-BPO group is consistent with the study of Park et al. (2021), who observed a significant increase in Holstein steers under HS, suggesting the susceptibility of these immune-related cells to HS, and suggesting consideration of the eosinophil count together with the basophil count as a potential biomarker to detect HS in cows. However, due to the limited number of studies investigating the effects of polyphenol supplementation on the hematological profile of dairy cows under HS, the mechanisms underlying the observed changes in blood platelets and eosinophils remain unclear. Thus, further research is needed to clarify the potential role and mechanism of action of polyphenols on platelet and eosinophil pathways.

Taken together, the differences observed between the 2 groups are related to the improved physiological, metabolic, and behavioral adaptation induced by Nor-Grape BP-O supplementation, which enhanced the ability of cows to cope with HS without altering rumen functionality.

CONCLUSIONS

The dietary inclusion of rumen-protected grape polyphenols offers a promising nutritional strategy to mitigate the adverse effects of naturally occurring HS in dairy cows. By improving thermoregulatory responses, reducing metabolic heat production, and supporting immune function, this supplement enhances overall cow performance during periods of environmental stress. The observed improvements in milk yield, immune modula-

tion, and inflammatory regulation highlight the potential of grape polyphenols as an effective and practical tool to promote resilience and productivity in heat-stressed dairy herds.

NOTES

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Nonstandard abbreviations used: A:G = albumin-to-globulin ratio; adj. = adjusted; ALP = alkaline phosphatase; AOPP = advanced oxidation protein products; AST = aspartate aminotransferase; CTR = control, standard milking cow diet; FRAP = ferric-reducing antioxidant power; GGT = gamma-glutamyl transferase; HB = heavy breathing; HS = heat stress; max = maximum; min = minimum; MCH = mean corpuscular hemoglobin; MPO = myeloperoxidase; MY = milk yield; NEFA = nonesterified (free) fatty acids; NG-BPO = diet supplemented with Nor-Grape BP-O (Nor-Feed); OD = optical density; PON = paraoxonase; ROM = reactive oxygen metabolites; ROMt = total reactive oxygen metabolites; RT = rectal temperature; SHp = thiol groups; THI = temperature-humidity index; Trt = treatment effect.

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