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Zootechnical evaluations of the adaptive capacity of native cattle breeds to climate change under different farming conditions

Tesi di Dottorato di Annalisa Amato

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Riassunto

I cambiamenti climatici stanno influenzando in misura sempre più crescente gli allevamenti, riducendo la produttività, compromettendo il benessere e la salute degli animali e causando perdite economiche. Infatti, nell'ottica di contrastare gli effetti negativi dell'aumento delle temperature, gli allevatori hanno l'esigenza di investire su strategie manageriali finalizzate all'adattamento ai cambiamenti climatici. Questi investimenti includono l'adeguamento delle strutture aziendali (implementazione di strutture per l'ombreggiamento), l'aumento dei costi operativi legati all'utilizzo di acqua (uso di sistemi di raffrescamento) e per l'energia elettrica (uso di impianti di ventilazione). In questo contesto l'allevamento bovino è fortemente impattato, in quanto le bovine ad alta produzione risultano particolarmente vulnerabili allo stress da caldo a causa della selezione genetica per l'elevata produzione di latte (che aumenta la produzione di calore metabolico) e dell'elevata ingestione alimentare (che comporta una maggior produzione di calore endogeno dal processo di digestione). Le ricerche attuali sullo stress da caldo si sono concentrate principalmente sulla razza Frisona, ma diversi studi hanno evidenziato una diversa risposta allo stress da caldo tra le varie razze ad alta produzione. In questo contesto, a partire dall' ipotesi che le razze locali possano mostrare una maggiore termotolleranza grazie all'adattamento ambientale, gli obiettivi di questa tesi sono stati di valutare la risposta allo stress da caldo nelle razze autoctone siciliane, in particolare Cinisara e Modicana, ed esaminare potenziali strategie nutrizionali per mitigare gli effetti negativi dello stress da caldo nelle bovine da latte.

La prima parte della ricerca ha valutato la termotolleranza nelle bovine Cinisare allevate in un sistema semi-estensivo durante il periodo estivo, attraverso il monitoraggio dell'indice temperatura-umidità (THI), la temperatura rettale (TR), la produzione lattea e la qualità del latte. I risultati hanno dimostrato che le bovine Cinisare sono in grado di tollerare livelli di THI più elevati rispetto alle razze commerciali come la Frisona. Infatti, TR e produzione lattea sono state influenzate solo quando il THI ha superato valori di 77. Al di sotto di questa soglia, TR, quantità e qualità del latte sono state solo lievemente condizionate dal THI. Inoltre, le bovine Cinisare hanno mostrato una TR più elevata durante le ore pomeridiane, ma comunque entro gli intervalli fisiologici, con una lieve variazione diurna. Questi risultati suggeriscono che la Cinisara possiede

un’efficace capacità di resilienza alle alte temperature, probabilmente attribuibile al suo patrimonio genetico e al lungo adattamento ambientale.

La seconda indagine di questa tesi si è concentrata sulla razza Modicana, valutando gli effetti dell’aumento del THI sulla produzione lattea e sui marker plasmatici del sistema ossidante-antiossidante. Considerando diverse classi di THI (55, 68, 71, 80), nelle bovine Modicane non sono state riscontrate differenze nella produzione di latte, né nella qualità del latte, ad eccezione della percentuale di grasso. Tuttavia, i metaboliti reattivi dell’ossigeno (ROM) sono aumentati significativamente, mentre paraoxonasi (PON), prodotti avanzati di ossidazione proteica (AOPP) e β-carotene hanno mostrato una significativa diminuzione. Allo stesso tempo, lo stress da caldo ha indotto una riduzione del glucosio plasmatico e degli acidi grassi non esterificati (NEFA) e un aumento dei monociti. I risultati hanno indicato che, pur essendo considerata una razza resiliente – che da un lato non ha mostrato differenze nella produzione lattea – può comunque andare incontro a stress ossidativo e metabolico con elevate temperature, evidenziando la necessità di adottare strategie che preservino la stabilità fisiologica durante eventi di stress da caldo.

L’ultima parte della tesi ha esplorato un approccio nutrizionale per mitigare gli effetti dello stress da caldo, integrando bovine di razza Pezzata Rossa con un estratto di vinaccia essiccata rumino-protetta (Nor-Grape BPO), ricca di polifenoli a basso peso molecolare. In condizioni naturali di stress da caldo (THI > 72), le bovine che hanno ricevuto il supplemento hanno mostrato performance e risposte fisiologiche migliorate rispetto a quelle che non hanno ricevuto il supplemento. Le bovine che hanno ricevuto il supplemento hanno infatti evidenziato una migliore termoregolazione, con TR più basse e riduzione della frequenza respiratoria. Anche le risposte immuno-metaboliche sono migliorate, con livelli plasmatici di zinco e mieloperossidasi più elevati, minore aptoglobina, una maggiore attività fagocitaria di neutrofili e monociti, e una modulazione delle citochine infiammatorie durante il picco di stress da caldo. Questi risultati hanno dimostrato che l’inclusione di Nor-Grape BPO rappresenta una strategia nutrizionale promettente per contrastare gli effetti negativi dello stress da caldo nelle bovine da latte.

Abstract

Climate change is increasingly impacting livestock farming by reducing animal productivity, compromising animal welfare and health, and causing economic losses. As a consequence, mitigating the negative effects of rising temperatures requires farmers to adopt several management strategies, such as upgrading farm facilities (e.g., fans, sprinklers, and shading structures), along with higher operating costs associated with increased water use for cooling and electricity consumption for ventilation systems. In this context, cattle farming is among the most affected, since high-producing cows are particularly vulnerable to heat stress (HS), due to the genetic selection for milk yield (which increases metabolic heat production), and due to the greater feed intake (more digestion-related endogenous heat). Current HS research has mainly focused on Holstein cows, but different studies have shown a different HS responses among distinct high producing cows breed. In this context, based on the hypothesis that local breed may have higher thermotolerance due to environmental adaptation, the aim of this thesis was to evaluate HS response in Sicilian local breeds, specifically Cinisara and Modicana cow and to evaluate potential nutritional strategies to mitigate the negative effects of HS in dairy cows.

The first part of the research assessed thermotolerance in Cinisara cows managed under semi-extensive systems during summer, by monitoring temperature-humidity index (THI), rectal temperature (RT), milk yield (MY), and milk composition. The results demonstrated that Cinisara cows were able to tolerate higher THI levels compared to high-producing breeds such as Holstein. Indeed, both RT and MY were affected only when THI exceeded 77. Below this threshold, RT, milk quantity and quality were only slightly affected by THI. Moreover, Cinisara cows exhibited higher RT during the afternoon hours, but within the physiological range and with a slight diurnal variation. These findings suggested that Cinisara has effective resilience to high temperatures, likely due to its genetic background and long-term adaptation.

The second part of this thesis focused on Modicana cows, evaluating the effects of increasing THI on milk performance and on oxidant-antioxidant plasma markers. Across different THI classes (55, 68, 71, 80), Modicana cows showed no differences in MY, and for milk quality, except for milk fat levels. However, reactive oxygen metabolites (ROM) significantly increased, whereas paraoxonase (PON), advanced oxidation protein products (AOPP), and β-carotene, significantly decreased. At the same time, HS reduced plasma glucose and non-esterified fatty acids (NEFA), and increased monocytes. The findings of this study indicate that, despite being considered a resilient breed – showing no significant reduction in MY – also local breed can experience oxidative and metabolic strain under high thermal load, highlighting the need to adopt strategies that preserve physiological stability during HS events.

The final part of this thesis explored a nutritional approach to mitigate HS effects by supplementing Fleckvieh cows with rumen-protected dry grape extract (Nor-Grape BPO), rich in low–molecular weight polyphenols. Under naturally occurring HS conditions (THI > 72), supplemented cows exhibited improved performance and physiological responses compared with cows without supplementation. Indeed, supplemented cows showed better thermoregulation, reflected by lower RT and reduced heavy breathing. Immune-metabolic responses improved as well, with higher plasma zinc and myeloperoxidase, reduced haptoglobin, enhanced neutrophil and monocyte phagocytic activity, and modulated inflammatory cytokines during peak HS. These findings demonstrated that inclusion of Nor-Grape BPO offers a promising nutritional strategy to counteract the negative effects of HS in dairy cows.

CHAPTER 1

1. General Introduction

1.1. The impact of climate change on livestock farming

The livestock industry plays an important role in the global economy. According to FAO (2009), there will be an expected increase of 70% in demand for animal products by 2050. The major concern of this increase is a combination between the projected future increase in global population – from 7.2 to 9.6 billion by 2050 (United Nations, 2013) – and the climate change phenomena, both of which poses a major challenge to meet future demands in animal products. Indeed, global temperatures have increased by 1°C relative to the beginning of last year, with an expected future increase of +1.5-2°C by the end of the 21st century (IPCC, 2023). Within this context, climate change can impact livestock farming in a negative way, both directly and indirectly. Indirect effects include changes in feed crop and forage production, water availability, land degradation and a decline in biodiversity (Rojas-Downing et al., 2017). The direct effects of climate change impact the animals ’thermoregulatory capacity leading to heat stress (**HS**). Heat stress is a serious issue in livestock production, as it negatively affects health, animal behaviour, fertility and physiology, with a greater risk of death in extreme HS cases. The severity of its impact depends on several factors such as species, breed, and production level, since each category has a different thermoneutral zone (TNZ), within which the animal's energy expenditure is minimal, constant, and unaffected by environmental temperature (Figure 1) (Nardone et al., 2006).

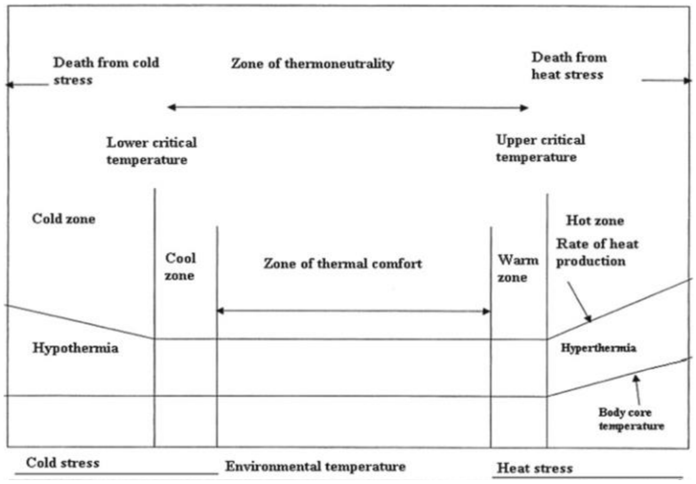

Figure 1. Thermoneutral zone scheme in the major livestock species (Kadzere et al., 2002)

In situations of HS, the different livestock species (e.g dairy cows, beef cattle, buffalo, sheep, goat, pigs and poultry) can react differently depending on the animal's sensitivity to high temperatures which is normally bound by the upper critical temperature.

It is noteworthy that the influence of TZN is not only related to the environmental temperature but is also correlated to relative humidity in the air. Indeed, high air relative humidity has a cumulative effect, since the ability to dissipate heat via evaporative cooling is significantly reduced. Other important environmental factors affecting the TZN are wind speed (enhancing the loss of body heat via the convection and evaporation pathways), precipitation (which can enhance heat loss by wetting and cooling the animals' coat through rain and snow), and solar radiation (which exposure can increase hyperthermia). Moreover, another important factor which is intrinsic to the animal rather than dependent on the environment, is coat colour and hair characteristics, which can influence the ability of absorbing solar radiation (e.g. in animals with black coat) as well as the ability to trap heat and moisture.

1.2. General response to HS

In general, when thermal comfort is compromised, and animals are exposed to temperatures beyond their TZN, homeostatic adaptation mechanisms are activated, the efficiency of which strongly depends on the interaction between the animal's intrinsic anatomical and physiological factors (Hoffmann, 2010).

With the onset of HS, the first mechanism to kick in is the attempt to dissipate heat through evaporative means, with conduction, convection and radiation, all of which depend on the temperature gradient between the animal and the environment (Collier and Gebremedhin, 2015). With the increase in ambient temperature, blood flow is redirected toward the skin surface to enhance heat loss via radiation; however, when the temperature gradient becomes minimal or even negative, these mechanisms become ineffective, and evaporation remains the only means of heat dissipation (Mayorga et al., 2019). Although animals in this first stage can be more or less efficient to adapt to adverse environmental conditions, under prolonged or extreme situations, the homeostatic mechanisms activated may impair the welfare status. The initial physiological response to HS includes the increase of water intake, body temperature, sweating, and the respiratory rate, with a concurrent decrease in feed intake (Horowitz, 2002). In particular, the increase in body temperature as one of the first response, is not influenced just by the increase in the environmental temperature, but also by the combination with the metabolic heat production. For this reason, some species and in particular the high producing animals are more susceptible to HS, since they have an intrinsic high metabolic rate and heat increment (Bagath et al., 2019).

1.3. Effects of heat stress on different livestock species

The sensitivity gradient of the main livestock species to high temperatures has been described in Figure 2 based on the available literature. Dairy cattle are considered among the most heat-susceptible livestock species, together with pigs and poultry, whereas beef, buffalo (Bubalus bubalis), sheep, and goats classify as lower to moderate sensitive to HS.

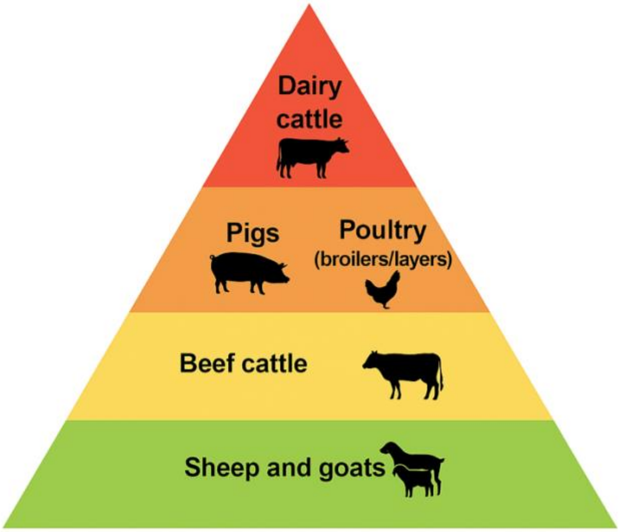

Figure 2. Ranking of heat stress response in different livestock species.

Pigs are particularly vulnerable to HS since they have few functional sweat glands, relatively small lungs and a thick layer of subcutaneous fat, all of which limit their ability to dissipate body heat (Black et al., 1993; Ross et al., 2015). For this reason, an increase in respiration rate is the most efficient way to dissipate heat in pigs (Collier and Gebremedhin, 2015). All these challenges impact first and foremost the daily feed intake, and thus growth performances, both of which decrease in order to minimise the metabolic heat production (Serviento et al., 2020). Previous studies also reported reduced growth in piglets and increase in neonatal mortality (Ross et al., 2015).

Due to the high genetic selection imposed on commercial broiler chickens and laying hens, these animals have high metabolic rates and as a consequence also generate high metabolic heat (Tallentire et al., 2016). Moreover, similar to the situation in pigs, poultry are unable to sweat, since they lack sweat glands, and thus dissipating heat mostly by panting (Vandana et al., 2021). The main impact of HS in laying hens manifest as a decrease in egg production, egg quality, yolk colour intensity, eggshell thickness, inferior nutritional value, and shorter egg shelf life (Deng et al., 2012; Lara and Rostagno, 2013), along with a decrease in meat quality in broiler chickens (Lu et al., 2007). The decrease in meat quality is in part related to the reduction in deposition of intramuscular fat in the breast muscle and in the deposition of proteins (Zaboli et al., 2019).

Domesticated buffaloes (Bubalus bubalis) are reared in many regions of the world, and for this reason they have evolved and gradually adapted to different climatic conditions. Although buffaloes are generally more tolerant to high temperatures and tropical conditions compared with those species previous mentioned, they are particularly sensitive to direct solar radiation due to their dark skin, sparse hair coat, and low sweating ability (Napolitano et al., 2023). However, they have developed adaptive mechanisms such as the deposition of a high concentration of melanin in the skin and the presence of numerous sebaceous glands, thus limiting the penetration of ultraviolet radiation into the dermis (Shafie, M.M., 1985) and, through the secretion of sebum to reflect solar rays (Shafie, M.M., 1970).

Compared with the previously mentioned species, dairy sheep and goats are well adapted to harsh environments and are therefore considered less susceptible to HS (Silanikove, 2000). Their greater ability to cope with HS is associated with them having lower basal heat production, increased saliva secretion, a larger absorptive mucosal surface area, a higher efficiency in recycling urea from the blood to the rumen compared with other grazers, and the ability to markedly expand the foregut volume when consuming high-fibre diets (Bernabucci et al., 2010). Although the effects of HS are generally less severe in these species, some studies have reported a reduction in dry matter intake with no significant impact on milk yield (Mehaba et al., 2021), while others observed decreases in growth performance, milk quality and quantity, and in immune function (Caroprese et al., 2008). Overall, although sheep and goats are considered more resilient, it is noteworthy that—even if to a lesser extent—HS still affects these species.

During HS beef cattle exhibit negative effects, showing reduced feed intake, lower nutrient assimilation and slower growth performance (O'Brien et al., 2010; Zhang et al., 2020), they are however more resilient when compared to dairy cows. This difference is mostly related to the fact that beef breeds have a lower metabolic heat production and a greater ability to tolerate higher temperatures before showing signs of HS. Indeed, as mentioned before, the higher metabolic heat production is associated with cow having a high milk production capability and as a consequence a capacity for a high daily feed intake with a relative high rumen fermentation, making dairy cows highly susceptible to HS (Polsky and von Keyserlingk, 2017). For this reason, high performing dairy cows commonly experience greater physiological, productive, and reproductive impairment than other livestock species. Considering the important role that dairy farming has within global food supply systems, investigating the mechanisms through which HS affects dairy cows is essential to identify effective strategies for mitigation and adaptation.

1.4. Heat stress in dairy cows

Dairy cows have a TNZ ranging between 5 and 25 °C (Roenfeldt, 1998), within which the animals remain in a condition of thermal comfort. When cows are exposed to temperatures outside this range, homeostatic adaptation mechanisms are triggered. However, the efficiency to counteract the negative effects is strongly dependant on the interaction between the animal's anatomical and physiological traits (Hoffmann, 2010). Although on the onset of HS, dairy cows start to adapt to adverse environmental conditions, under long-term or extreme situations, the homeostatic mechanisms involved may ultimately compromise animal welfare (Figure 3).

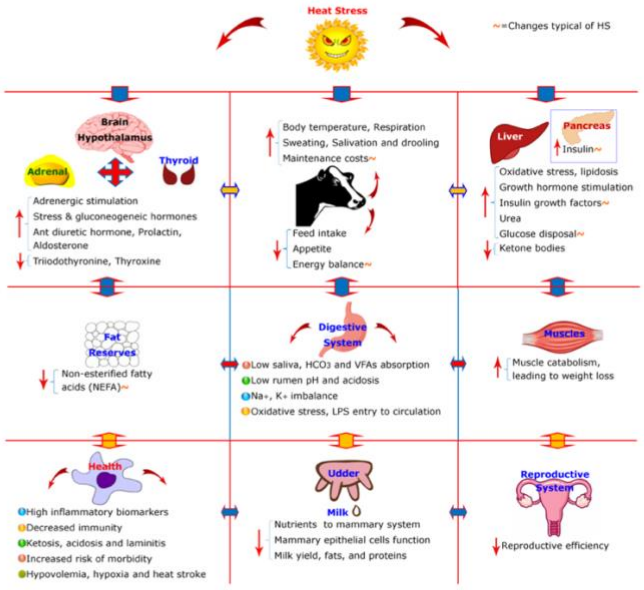

Figure 3. Overview of the physiological and biochemical alterations induced by heat stress in different systems of dairy cows (Sammad et., 2020)

The initial response to HS involves the increased intake of water, sweating, and higher heart and respiratory rates, along with a decrease in feed intake (Horowitz, 2002). In the long term, these factors lead to an overall reduction in milk yield, reproductive performance, growth, welfare and health status, and alterations in immune response (Osei-Amponsah et al., 2020), all having an adverse effect on farm profitability. A 2022 report (ANAFI, 2022) estimated that in Italy, economic losses of approximately €0.72 per cow per day occur during the summer months due to decreased milk production. The decrease in milk production ranges from an estimated reduction of about 17 to 53% (Garner et al., 2017), with high-yielding cows being more susceptible due to the positive correlation between milk production and body metabolic heat (Spiers et al., 2004). Nevertheless, the decline in milk yield (MY) is also associated with metabolic alterations that occur during HS. The reduction in MY is in part linked to the decrease in dry matter intake (DMI), which plays a central role in the negative energy balance that develops (Sammad et al., 2020b). Reduced DMI leads to an insufficient energy supply, resulting in the mobilisation of body fat reserves to compensate for the gap between dietary energy intake and the energy required for milk production (Bauman and Bruce Currie, 1980). Indeed, dairy cows under HS tend to use carbohydrates, such as glucose, as their primary energy source, prioritizing glucose utilization by body tissues over milk synthesis (Baumgard and Rhoads, 2012). Apart from glucose metabolism, other metabolic mechanisms are also impacted. Joo et al. (2021) investigated the mechanisms of metabolic alterations caused by HS through blood parameter analysis and reported reductions in total cholesterol, LDL cholesterol, proteins, albumin, blood urea, creatine kinase, calcium, sodium, potassium, chloride, and magnesium.

Several studies have reported a range of negative outcomes of HS on reproductive function, including reduced oestrus detection rates (Collier et al., 1982), altered follicular function (Schüller et al., 2017), and early embryonic death (Lannett and Hansen, 1997), ultimately leading to infertility (Negrón-Pérez et al., 2019). The digestive process is also compromised due to the redistribution of blood flow to the periphery, to enhance heat dissipation, leading to reduced gastrointestinal blood supply (Sammad et al., 2020b). The immune system is also jeopardised due to cortisol release, altering cellular immune functions by inhibiting the expression of genes involved in T cell activation and cytokine production (Bagath et al., 2019). Such immune impairment represents a major concern, as animals become more susceptible to secondary diseases.

To assess HS in ruminant farming, the temperature-humidity index (THI) is commonly used as a reference of an objective indicator to estimate the animal's thermal comfort or discomfort level. The THI is calculated using the following equation:

$THI = (1.8 \times T + 32) - [(0.55 - 0.0055 \times RH) \times (1.8 \times T - 26)]$ (Dikmen et al., 2008)

[T = air temperature (°C) and RH = relative humidity (%)]

Although this index provides an objective measure of the thermal comfort of dairy cow, several studies have reported conflicting thresholds for defining the breakpoint at which animals can be considered under HS. Commonly, a THI value of around 72 is considered the upper threshold for heat stress in Holstein cows (Heinicke et al., 2018), as MY begins to decline beyond this point. However, studies (Mader et al., 2006) have reported a wide range of THI threshold values, from 60 to 74, depending on breed, environmental conditions, and the production traits being evaluated. For instance, lower thresholds were suggested for Holstein cows, while higher ones were identified for more heat-tolerant breeds such as Brown Swiss (Maggiolino et al., 2020). The variabilities in these findings suggest that THI cannot be considered as a strictly objective indicator of HS, since it is strongly influenced by multiple biological, genetic and environmental factors. Thus, there is the need to investigate and understand the variability of this index for each breed, in order to adopt breed specific different prevention and mitigation strategies to counteract the negative effects of HS.

This literature review indicates that proper management dairy cows during HS is essential to prevent the negative consequences on animal health, welfare and performance. Therefore, providing a farming environment equipped with appropriate management systems that ensure both thermal and husbandry comfort is of fundamental importance. In the next paragraphs of this thesis, will be summarized the most relevant mechanisms adopted by dairy cows to counteract HS and the possible mitigation strategies reported in the literature.

1.5 Behavioural and physiological responses to heat stress

1.5.1 Sweating and panting

When environmental temperature exceeds 25°C, cows start to promote heat dissipation, which can be enhanced through conduction, radiation, convection and evaporation. The ability to dissipate heat can be influenced by several factors, such as the density of hair follicles, sweat glands, nerve fibres and capillary surface (Taneja, 1956). The latter is pivotal for thermoregulation, as elevated body temperature enhances blood perfusion in capillary beds (Hales et al., 1978), thereby promoting heat loss through the skin and respiratory tract. The mechanisms adopted depends on the environmental temperature; hence, when environmental temperatures are low, heat loss occurs mainly via radiation and convection, whereas at higher temperatures, vaporization becomes the primary mechanism (Kadzere et al., 2002). In particular, heat loss via skin through vasodilatation depends on the temperature gradient between skin and air, and is positively related to the sweating rate (Blazquez et al., 1994). About 75-85% of evaporative heat loss in cattle comes from sweating (Brown-Brandl, 2018), but its effectiveness can also be influenced by wind speed, humidity, density and activity of sweat glands and respiratory rate (Shephard and Maloney, 2023). Aggarwal and Upadhyay (2013) report that at an environmental temperature of 40°C, there is a maximum evaporation from skin surface of about 150g/m^2/h. However, an important aspect to consider is also the role of relative humidity (RH) in modulating the animal's thermoregulatory efficiency. Since sweating represents one of the primary mechanisms for heat dissipation, elevated RH markedly reduces the evaporative gradient between the skin surface and the surrounding air, thereby impairing evaporative heat loss. As a consequence, the effectiveness of sweating is compromised under high-humidity conditions, exacerbating the risk of HS even at comparable ambient temperatures (Jo and Lee, 2025).

On the other hand, about 15% of the endogenous heat is dissipated by the respiratory tract through the increase in respiration rate (RR) (McDowell et al., 1976). Usually, RR is measured by counting the number of breaths per minute through the flank movements, and its physiological range is between 26 and 50 breaths per minute (Becker et al., 2021). However, in recent years, the use of accelerometer-based sensors has been increasingly adopted to monitor the animal's respiration pattern in a non-invasive and autonomous way, in real time and on a continuous basis (Islam et al., 2020). In particular, these innovative systems are important to detect heavy breathing (HB) due to the acceleration in RR; a strong indicator that the animal is experiencing HS (Polsky and von Keyserlingk, 2017; Brown-Brandl et al., 2006). In fact, HB enhances the evaporative cooling through the mucosa membranes of the respiratory tract. In dairy cows, a respiratory rate exceeding 80 breath/minute is generally considered a sign of severe HS (Berman, 2005). In general, a condition of HB may represent a challenge since it can contribute to rumen acidosis, due to the higher exhalation of CO2 and thus its lower levels in blood, which prompt the kidneys to excrete bicarbonate to maintain the 20:1 ratio of bicarbonate-to-CO_2 (Sammad et al., 2020b). Consequently, less bicarbonate is present in saliva to buffer and stabilize rumen pH. Moreover, panting cows often drool, further reducing salivary flow. The combined reduction in salivary bicarbonate concentration and volume increases the susceptibility of heat-stressed cows to both subclinical and acute rumen acidosis (Kadzere et al., 2002; Sammad et al., 2020b).

1.5.2. Body temperature and heart rate

Body heat production is associated with milk production, physical activity, feed intake and digestive process. A physiological body temperature results from the balance between heat gain and heat loss, which in cows exposed to HS is the combination of environmental heat accumulation and the inability to effectively dissipate metabolically produced heat (West, 2003). The impairment of this imbalance increases core body temperature. In dairy cattle, the core body temperature ranges from 38.0 to 39.3 °C (Gaughan, 2002), and the standard method of detection is from vaginal or rectal measurements, which represent the gold standard for its detection. The importance of its detection is related to its association with the temperature of very important organs such as the brain and the heart (Farooq et al., 2010). An increase in rectal temperature (RT) above 39°C, indicates that cows are undergoing hyperthermia (Kadokawa et al., 2012), impacting both welfare of the animal, and the consumption of feed and milk production, both of which decrease in order to retain low the core body temperature. Indeed, Rejab et al., (2016) estimated that when RT is above 38.2°C, a reduction in milk yield of 1.1 kg/d will occur for each point of degree above this threshold.

The increase in RR, together with the effort to enhance heat dissipation by increasing blood flow to the body surface, leads to an increase in heart rate (HR). For this reason, heat exposure results in elevated cardiac output, thus HR is another important physiological parameter to assess the HS condition in dairy cows. Indeed, previous studies observed an increase in the rate of heart beats of

15% (Giannone et al., 2023), which is normally between the range 54-84 bpm (Detweiler, D.K. and Erickson, H.H., 2004).

1.5.3. Drinking behaviour and feed intake

During HS, the consumption of water increases with the rise in environmental temperature, in an attempt to improve the evaporative cooling and to offset the water losses through sweating and panting (Kadzere et al., 2002). In thermoneutral conditions, cows producing 41.5 kg of milk per day consume approximately between 70 to 100 Liters of water daily (Kononoff, P.J., and K.J. Clark, 2017). However, during summer, water intake (WI) is further affected by DMI and MY, with higher-yielding cows showing greater requirements.

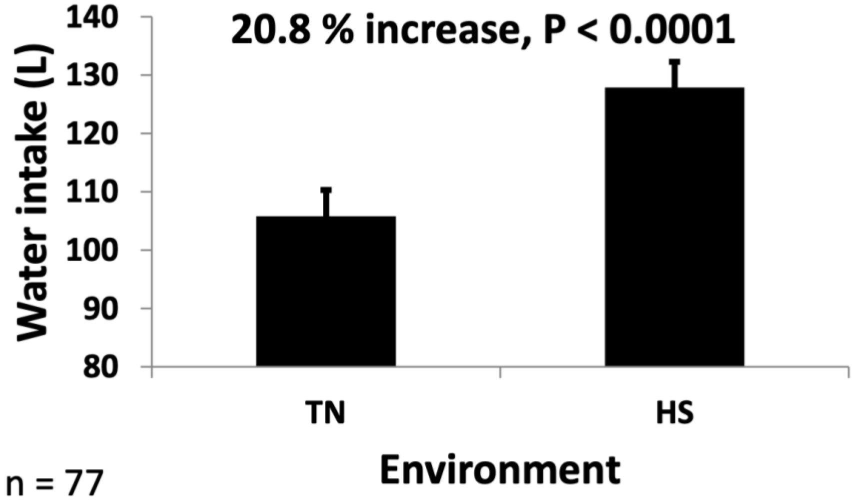

Figure 4. Mean water intake responses to an increase in THI from 57 (TNZ) to 73 (HS)(Collier et al., 2019)

In this context, previous studies demonstrated an increase in WI of 20.8% (Figure 4) (Collier et al., 2019) or even twofold when temperature rise from 15 to 33°C (Beatty et al., 2006) and an increase of the total daily time spending drinking from 0.3 h (THI 56.2) to 0.5 h (THI 73.8) (Cook et al., 2007).

The decrease in feed intake during HS is an important problem since it results in low milk yield (and thus economic losses) for a potential decrease of about the 50% (Wheelock et al., 2010a). When dairy cows are within the TZN, they normally consume from 12 to 15 meals per day, whereas during HS the feed frequency decreases from 5 to 3 eating episodes per day, resulting in larger meals that could compromise gut health (Kadzere et al., 2002). For this reason, animals tend to shift their feed behaviour by eat more during the night hours, when non-evaporative heat loss to the environment is more efficient (Aharoni et al., 2005). Moreover, the decline in feed intake can be related not only to the decrease in appetite in dairy cows, but also as a strategy to minimise the production of heat during ruminal fermentation and nutrient metabolism. A previous study reported a decrease in DMI of 0.51 kg for every unit increase in THI above 73 (West, 2003), highlighting a significative impact in the amount of feed consumed.

The decrease in feed intake is also related to the stimulation of hypothalamic axis through the increase in leptin and adiponectin levels (Belhadj Slimen et al., 2016), leading to marked alterations in blood metabolites and glucose levels (Guo et al., 2017a). The consequent reduction in nutrient absorption contributes to a decrease in energy intake and induces metabolic and hormonal adjustments (Bernabucci et al., 2010). The imbalance between reduced high energetic demand for milk production compromises body weight, leading to its decline, and results in a negative energy balance (NEBAL)(Sammad et al., 2020b). These alterations in energy status induce significant modifications in carbohydrate, lipid and protein metabolism under HS conditions.

1.5.4. Rumination time and rumen microbiota

One of the consequences for the reduces DMI, is the decrease in eating time and hence less time spent in rumination. Previous studies by Ramòn-Moragues et al., (2021) reported a decrease in rumination time as THI increases, with a reduction of 2 min for every unit of THI beyond 76. This decrease may slow the passage of digesta through the gastrointestinal tract, limiting the rumen's capacity for additional feed intake and ultimately decreasing DMI (Church, 1993). Moreover, rumination time can be also compromised by the changes in the time spend standing, as a strategy adopted by the animal to enhance heat dissipation. Although the standing position could enhance heat dissipation trough convection, radiation and water loss, the effect of standing instead of lying down can compromise milk production since blood flow to the udder is reduced (Rulquin and Caudal, 1992), and animal health as risk of lameness increases (Cook et al., 2004). Similar to RR, rumination time can be easily recorded using an accelerometer-based technology offering continuous, less intrusive monitoring with the use of collars, ear tags, or even ruminal boluses

The high incidence of rumen acidosis during HS (Yadav et al., 2013) and thus changing in rumen pH influences the profile of microbial population. Previous studies have observed reduced cellulolytic and fibrolytic species such as *Fibrobacter succinogenes*, *Ruminococcus flavefaciens*, and *Prevotella ruminicola*, and an increase in lactic acid–producing bacteria such as *Streptococcus* and *Enterobacteriaceae*, which result in an increase in lactic acid (Correia Sales et al., 2021). The increase in lactic acid production in the rumen may enhance its absorption in the bloodstream, potentially exerting negative effects on animal health (Newbold et al., 1987). Moreover, the excessive lactate accumulation will reduce the acetate levels and increase the levels of propionate and butyrate concentrations, thereby disrupting the acetate-to-propionate ratio and overall rumen fermentation (Wang et al., 2012).

1.5.5. Fertility and reproduction

The increase of environmental temperature is also associated with the reduction of fertility and reproductive performances of dairy cows (Rensis and Scaramuzzi, 2003). Previous studies observed weakening of the dominant follicle activity, allowing more medium-sized subordinate follicles to persist (Wolfenson et al., 1995; Wilson et al., 1998). Moreover, during HS oestrus is not detected in the 80% of cases, mainly due to the shortness of duration and intensity of oestrus signs (De Rensis et al., 2015). It is worth noting that a high environmental temperature can also compromise the intrauterine environment, increasing the uterine temperature and reducing its blood flow (Sammad et al., 2020a). The high intrauterine temperature creates a suboptimal environment for oocyte development, influencing also sperm, and embryo survival (De Rensis et al., 2015). Most pregnancy losses occur during the early embryonic period (Days 8–17), impairing embryonic development and reduces interferon-tau production, essential for corpus luteum maintenance and pregnancy recognition (Inskeep and Dailey, 2005). The major concern regarding the impact of HS on dairy cow fertility are summarized in Figure 5. The effects of HS are not confined to the period of exposure, which may vary in duration, but can have long-lasting consequences affecting both the offspring and the cow's subsequent productive and reproductive performance (Ouellet et al., 2020).

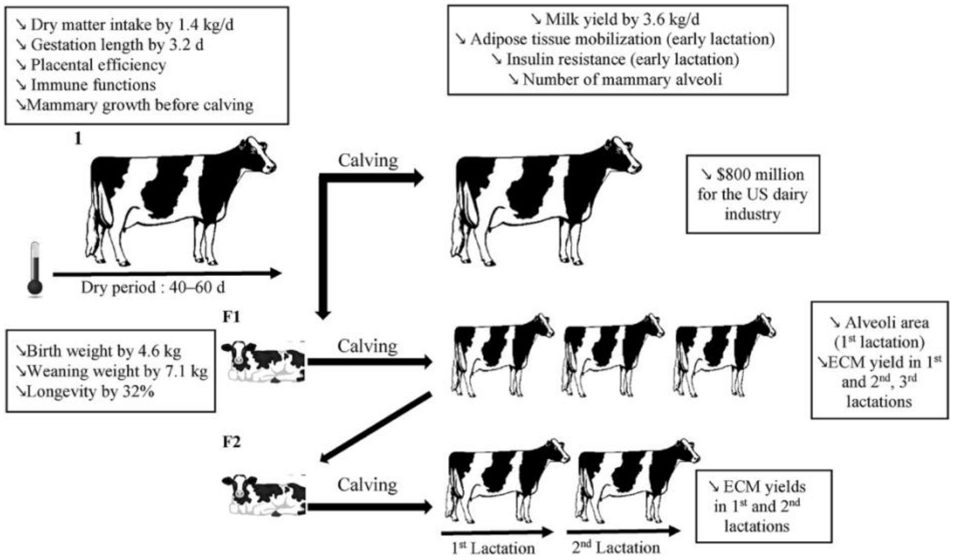

Figure 5. Summary of the detrimental effects of late-gestation heat stress on performance across generations (dam, daughters, and granddaughters) (Ouellet et al., 2020)

This study was observed that HS compromised mammary gland involution in the dam, reduced immune function, and altered metabolic adaptations at the onset of lactation. On the other hand, calves exposed to intrauterine HS are typically born with lower body weight, impaired immune fitness, and show long-term consequences, including reduced survival, lower milk yield across lactations, and shortened productive longevity. Moreover, the exposure of the embryo to HS in utero can result in epigenetic variations that manifest in the phenotype during adult life and can be inherited by the next generation (Huber et al., 2020).

1.5.6. Oxidative stress and immune function

Among the physiological mechanisms adopted during HS, oxidative stress has a major impact on cow welfare and plays a pivotal role in the cascade of events, compromising the animal's immune system. Oxidative stress results from an imbalance between the increasing production of free radicals (such as the reactive oxygen species, ROS) and the antioxidative defences, which compromise the metabolism and physiology of the animal (Trevisan, 2001). Reactive oxygen species, such as superoxide anions, hydrogen peroxide, and hydroxyl radicals, are byproducts of aerobic metabolism with both physiological and harmful roles (Halliwell, 2007). At moderate levels, ROS are important for the physiology of the organism since they contribute to immune defence and cellular signalling (Vajdovich, 2008). However, when ROS production is excessive, they can damage lipids, proteins, and DNA, leading to metabolic dysfunction and cell death (Davies, 1995). Although under physiological conditions, the natural antioxidant system neutralises the production of free radicals and ROS, during HS dairy cows are unable to counteract their excessive production and maintain redox balance. Indeed, under HS, the overproduction of free radicals and ROS and mitochondrial dysfunction will disrupt cellular homeostasis, leading to increased apoptosis of the mammary epithelial cells and reduced secretory capacity (Guo et al., 2021).

Moreover, oxidative stress can contribute to insulin resistance, which interferes with the insulin–mTOR signalling pathway, a central regulator of protein synthesis in the mammary gland (Guo et al., 2021). Safa et al. (2019) reported that oxidative stress occurs mainly due to lower levels of total antioxidant capability (T-AOC) and superoxide dismutase (SOD) and higher lipid peroxidation.

Due to its multiple negative effects of HS on the physiological status of dairy cows, the immune function is compromised increasing the cow's susceptibility to disease. This can occur in part due to the stimulation of the hypothalamic–pituitary–adrenal-axis leading to an increase in the secretion of glucocorticoids, which suppresses pro-inflammatory cytokines such as IL-4, IL-5, IL-6, IL-12, IFN-γ, and TNF-α through the inhibition of the p38 MAPK pathway (Abraham et al., 2006; Bagath et al., 2019).

Compromission of the immune function is also related to the decrease in the weights of immune organs, like the thymus and spleen (Ahmed, 2017).

Both cell-mediated and humoral immune responses are compromised during HS, as reflected by reduced lymphocyte proliferation, impaired neutrophil function, and altered oxidative burst activity (Dahl et al., 2020). The decrease in the proliferation of immune cells and cytokine production together with the migration of lymphocyte to the udder, increases the incidence of production diseases such as mastitis and metritis in dairy cattle (Steele, 2016), particularly during hot seasons when pathogen load is also higher. A recent study conducted by Dahl et al. (2020) reported that HS affects the immune system of dairy cows throughout its life cycle, from the in utero stage up to the dry cows. Indeed, the exposure during late gestation reduces the colostrum immunoglobulin transfer to calves and weakens the passive immunity of offspring. In dry cows, HS impairs adaptive responses, such as antibody production following vaccination, and these effects persist into early lactation, compromising innate immune function and overall productivity.

1.5.7. Milk production and Quality

As a strategy to reduce the metabolic heat production and counteract hyperthermia dairy cows decrease DMI, and thus milk yield (MY). However, the decrease in MY is only in partly (35-50%) related to DMI (Baumgard and Rhoads, 2012). The combination of the decrease in DMI together with the increase in nutrient requirements for energy maintenance, reduce nutrient availability in the mammary gland (Wang et al., 2010).

Although previous studies demonstrated that the MY decline in Holstein occurs at a THI of 72 (Armstrong, 1994), more recent research indicated that this threshold has been reduced to 68 (Tao et al., 2020). This shift in sensitivity over the years is mainly related to the strong genetic selection imposed for higher milk production, making high-yielding cows more susceptible to HS (they must dissipate more heat). Previous studies have established that the increase in MY from 35 to 40 L/d reduces the threshold temperature by 5°C (Berman, 2005). For this reason, this threshold is general and can change among different breeds and production levels. Indeed, previous studies demonstrated that MY decreased by 0.69 kg in Holsteins and by 0.45 kg in Jerseys for each unit increase in THI, suggesting that breeds under higher selection pressure are more affected by HS (West et al., 2003). Another important reason causing the decline of MY is the impairment of mammary gland involution (Wohlgemuth et al., 2016). During the peripartum period there is an important regeneration process of the mammary gland, where there is a regulated and programmed cell death of old epithelial cells to generate new epithelial cells to renovate mammary gland occurs (Capuco et al., 1997; Menta et al., 2022). For this reason, attention should also be given to dry cows to prevent the negative effects of HS during the subsequent lactation.

Milk composition is also influenced by HS. Alteration in protein yield when THI is above 74 (Maggiolino et al., 2020) compromises milk coagulation properties with a carryover effect on cheesemaking. Changes in milk proteins are due to changes in DMI; however, as for MY, this is not the only contributing factor. Indeed, the reduction of amino acid circulation causes the reduction of casein in the mammary gland (with a marked decrease of both αs2- and β-caseins) and, as a consequence, decreased milk protein synthesis (Guo et al., 2017a). Also lipid composition is altered during HS, through the reduction of triacylglycerol (TAG) which contains mainly short- and medium-chain fatty acids and a higher content of long-chain fatty acids, as reported by Liu et al. (2017). This shift increase the viscosity and melting point of milk fat, potentially affecting the processing characteristics and physical properties of the subsequent dairy products (Liu et al., 2017). A previous study observed changes in the fatty acid profile, with a 13-14% increase in saturated fatty acids (SFA) and a decrease of total monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA) and conjugated linoleic acid (CLA) (Saroj et al., 2017).

Moreover, the decrease in DMI prompts the utilisation of glucose from the body reserves. As a consequence, lactose synthesis is also reduced since mammary gland does not receive enough glucose (Sammad et al., 2020b).

1.6. Heat stress mitigation strategies

The review so far focused the most investigated and documented negative effects of how different perspectives influence the dairy cows' health during HS. Over the years, as the issue of climate change has become prominent, the need to identify strategies to prevent or mitigate the adverse impacts of HS has grown significantly, encouraging researchers to explore innovative approaches. In the following section of this chapter, the main mitigation strategies reported in the literature will be discussed.

1.6.1 Animal housing management

One of the most adopted strategies to manage HS in dairy farms is the use of fans, spray cooling and ventilation. Indeed, in hot weather, cooling systems are important to maintain an adequate microclimatic condition to enhance the efficiency of heat dissipation mainly through convection. In this context, the use of forced ventilation systems for example, increases convective heat loss, in addition to increasing air flow and exchange.

Berman et al. (2006) showed that while evaporative cooling reduces ambient air temperature, its effectiveness diminishes as humidity rises. This technology becomes very limited when the air's relative humidity exceeds the 45% mark. In this study, cows exposed to a low air speed (0.3 m/s), and high humidity (55-75%) reached high stress levels, in particular with reduced respiratory heat loss, at an ambient temperature of around 31-34 °C. When the cows were exposed under higher air speed (1.5 m/s) and full body exposure, humidity showed less of an effect on respiratory heat loss. This finding highlights how air movement and a reduced body surface exposure are important stressors, suggesting that improving air speed in the proximity of the animals will have mitigation effects on HS.

The importance of ventilation has been evidenced in a recent study in calves validating the use of positive pressure tube ventilation (PPTV) (Guenther et al., 2025). This system distributes forced air evenly throughout the housing area, helping to lower body temperature and respiratory rate, and consequently improving animal comfort and growth performance.

As demonstrated by previous studies, the use of spray cooling is essential to lower the body temperature and RR in both milking and dry cows (Chen et al., 2013; Ouellet et al., 2021). These technologies are usually programmed to activate based on a previously established setup, related to the THI level or ambient temperature. Moreover, as reported by Tresoldi et al. (2018), a different spray duration (short vs. long) has different effects on skin temperature and RR. Short spraying episodes lowered skin temperature immediately but have a limited impact on body temperature, while longer spraying durations (≥ 1.5 min) significantly decreased RR and body surface temperature. These findings highlight that optimising the duration of spraying episode is essential to enhance the efficiency of the sprinkler cooling systems, while ensuring animal comfort and minimising water use. Although these cooling methods are generally effective in reducing heat load, attention should be paid to water consumption and the potential over-wetting of the bedding, which may negatively affect hygiene and cow comfort. Therefore, the combined use of ventilation and sprinkler systems is usually recommended to enhance cooling efficiency while limiting excessive moisture accumulation.

Another important environmental strategy which can be adopted to minimise HS, is to give animal access to sunshade to reduce heat gain from solar radiation. The importance of the use of shade was previous evaluated by Silanikove et al., (2009) who highlighted that their use in dairy cows exposed in a hot environment can reduce the radiant heat load by about 45%. Another study reported that giving cows access to shade will reduce the RR by 67% when compared with cows without shade (Kendall et al., 2007). For this reason, providing a natural or artificial shade to cows in a free stall barn setup will protect the animals from the solar radiation, and helping them to better dissipate heat. Several studies have demonstrated that the type of shade material can also strongly influence the microclimate within animal shelters. Singh et al. (1989) reported significant differences in THI among sheds with thatch, asphalt, and asbestos roofs, with thatch providing the greatest thermal protection during the hottest hours.

Kamal et al. (2016) evaluated how different shade roof materials (thatch, agro net 60% light diffusion, asbestos with canvas, and natural tree shade) affected behavioural, biochemical and hormonal responses. In particular, they found that agro net provided the most favourable micro-environment (lower THI) and supported better behaviours (increased feeding, rumination, resting and sleeping) compared to other materials. Overall, these studies highlighted how the selection of the best suited shading material can play a significant role in alleviating HS. Nevertheless, when RH is excessively high, the efficiency of these cooling strategies is inherently constrained (Berman, 2009). As mentioned before, since evaporative heat loss represents a major pathway for thermal dissipation in cattle, elevated humidity impairs evaporation, thereby reducing the effectiveness of sprinkler and ventilation systems and potentially exacerbating heat load.

1.6.2. Feeding supplementation

Among the different management strategies, the inclusion of essential nutrients is a practical and low-cost approach to reduce the impact of HS in dairy cows. Since cows under HS generally tend to decrease DMI, it is important to provide a properly balanced diet which can sustain the animal to maintain milk production and support its health and welfare. For this reason, it is important to implement a feeding management regime that will provide a balanced diet of the animal. In this context, previous studies (West et al., 2003) demonstrated that diets rich in fibre (NDF=42% of DM) decreased the cow performance and increased body temperature when compared to cows fed a diet with lower fibre content (NDF=30% of DM). A reduction in dietary forage and a corresponding increase in concentrate can enhance net energy intake, as concentrates are associated with lower heat production during digestion (Conte et al., 2018). Consequently, decreasing the forage-to-concentrate ratio may improve nutrient utilisation efficiency.

While it is important to limit metabolic heat production through dietary strategies, it is important to ensure an adequate protein intake, since amino acids are impacted during HS, due to the suppression of RNA transcription and translation processes (Sonna et al., 2002). Moreover, the decrease in amino acids is also related to their withdrawal and use by the liver for the production of heat-shock proteins and acute phase proteins (Ríus, 2019), thus reducing their availability for use in the mammary gland (Rhoads et al., 2009a).

For this reason, dietary inclusion of essential amino acids can help mitigate the negative effects on milk protein synthesis, immune function and metabolism during thermal stress. Specifically, methionine has gained particular attention, since it is an essential amino acid that plays a rate-limiting role in milk protein synthesis (Schwab and Broderick, 2017). In a recent study, rumen-protected methionine supplementation to transition cow, counteracted the reduction of blood glucose levels at calving and milk protein content in the dams, while lowering the RT in their calves (Davidson et al., 2024). Choline supplementation has also shown to have positive effects in heat stressed dairy cows, indeed, Zhou et al. (2017) observed an antioxidant effect through the regulation of lipid and energy metabolism, and McFadden et al. (2020) observed a better inflammatory response.

While providing protein supplementation in the diet of dairy cows is an important focus, it is also crucial to consider the crude protein content of the diet, which can contribute to an increase in metabolic heat production (Conte et al., 2018).

Among the different types of feeding supplementation, minerals play a crucial role, as they are essential micronutrients that cannot be synthetized by the body, and therefore need to be incorporated in the diet. Moreover, their supplementation is important during HS, not only because their demand increase (many minerals are lost through sweating), but also since there is an increase in the demand for minerals to enhance thermoregulation (Guesine et al., 2023). Important minerals include sodium, potassium, magnesium, zinc, selenium, copper, and manganese, as well as chromium. For example, Weng et al. (2018) demonstrated that dietary supplementation of Zn supports immunity, metabolism and mammary epithelial barrier function in lactating dairy cows under HS. Moreover, Sheikh et al. (2017), observed a decline in IL-6 and an improved immune response in periparturient dairy cows under HS supplemented with Zn. Another important mineral is chromium, which improves the energy metabolism trough the reduction of NEFA, increased insulin concentration and reduced cortisol levels (Soltan, 2010). Selenium supplementation did not have significant effects on milk production, but showed positive effects in the percentage of milk fat and an improvement in mammary gland health (Oltramari et al., 2014).

Apart from minerals, vitamins are also an important component in cows' diet formulation, since most vitamins are not synthesized in sufficient amounts by the body. Moreover, their deficiency may be associated with a decrease in the immune response and an increase in the incidence of diseases. In this context, niacin (vitamin B3), when converted into nicotinamide, can support animals during HS, taking part in anaerobic glycolysis and tissue respiration (Guo et al., 2017b). Supplementation with niacin in dairy cows exposed to HS, has been reported to promote skin vasodilation and improve peripheral blood circulation (Di Costanzo et al., 1997). Furthermore, supplementation with a combination of rumen-protected capsule containing niacin, K2SO4 and vitamin C for 42-days had beneficial effects on lactation performance and immunity, lowering IL-6 concentration, and alleviating the inflammatory response during HS (Guo et al., 2017b).

Plant-derived polyphenols are well-known for their antioxidant properties and their ability to reduce ROS levels, enhancing antioxidant defenses. Among plant sources, green tea (*Camellia sinensis L.*) represents a rich source of catechins and other bioactive compounds with antioxidant, antimicrobial, and antiprotozoal activities (Kolling et al., 2018). For this reason, its supplementation in dairy cows diets has been shown to reduce oxidative stress, enhance the activity of antioxidant enzymes, and activate the Nrf2/heme oxygenase-1 (HMOX1) pathway (Gessner et al., 2020). Similarly, *Capsicum* extracts have demonstrated beneficial effects by modulating inflammatory and oxidative responses, in particular through the lowering of cortisol and the haptoglobin levels (Oh et al., 2017).

Another potential supplement rich in polyphenols is grape seed, which has shown to have positive effects on milk production in dairy cows during the transition period (Gessner et al., 2015). However, as shown in Chapter 5 of this thesis, supplementation with rumen-protected dry grape extract in dairy cows under HS resulted in enhanced immune function, as evidenced by greater neutrophil activity and an improved inflammatory response, reflected by an increase in the concentration of plasma Zn and globulin.

1.6.3. Genetic selection and local breeds

The genetic approaches recently adopted to ameliorate the impact of HS, include the identification of genes associated with heat tolerance and the selection of heat tolerant breeds. Indeed, heat tolerant breeds, like indigenous dairy cows breed, can maintain milk production while coping with HS, thanks to physiological and genetic adaptation (Rojas-Downing et al., 2017). Although these breeds are usually characterized by a lower production potential, they are capable of maintaining it at a stable level, while high producing cows tend to submit to HS. Furthermore, local breeds thrive during the hot season with limited pasture availability and water scarcity (Sejian et al., 2018), thus proving their suitability for harsh and resource-limited environments. According to a review by Bernabucci et al., (2010) there are important differences between acclimation, acclimatization and adaptation, defining different levels of response to HS:

- **Acclimation** refers to short-term physiological or behavioral adjustments that occur within an animal's lifetime in response to controlled changes in the environment;

- **Acclimatization** involves similar adjustments but occurs naturally, for example across seasons or different geographical areas;
- **Adaptation** represents long-term changes that reduce the impact of HS and may result either from phenotypic plasticity or from genetic selection over generations.

In this context, local breeds represent the outcome of long-term adaptation, due to the natural selection of specific genes and phenotypic traits [e.g the coat colour, reduced metabolic heat production, improved water efficiency, better ability to dissipate heat via panting and sweating (Gaughan et al., 2009)].

An early and ancestral form of adaptation occurred between Bos taurus and Bos Indicus cattle. In fact, several studies have demonstrated that due to genetic adaptation, Zebu cattle have acquired thermotolerant genes and thus have a better degree of thermotolerance when compared with Bos taurus cattle (Hansen, 2004).

One of the first studies conducted on the molecular response to HS revealed that Zebu cattle can activate heat shock transcription factor 1 (HSF1) and the upregulation of heat shock proteins (HSPs)(Mehla et al., 2014). Moreover, this study also reports that the genes related to lipid metabolism, protein synthesis and immune function were suppressed and downregulated, insinuating that an energy conservation strategy is adopted by Zebu cattle. Another study by Edea et al. (2018), provided strong evidence of divergent selection pathways between the taurine and zebu cattle populations, highlighting that the two populations were subjected to different evolutionary pressures. In this study, several heat tolerance candidate genes were identified as candidates for heat tolerance (e.g., HSPA4, HSF1, EIF2AK4, CMPK1), pigmentation and solar radiation response (ERBB3, MYO1A), immune function (PIK3CD, AKIRIN2), and fertility (ID3, PSPC1). These findings demonstrated that Zebu and other locally adapted breeds have undergone a genetic selection process favouring traits that enhance thermotolerance and resilience in hot and resource-limited environments, whereas taurine breeds have been shaped by selection in more temperate climates.

Another important role in thermoregulation has been attributed to the slick hair coat. Mariasegaram et al. (2007) identified a mutation in the PRLR gene, known as SLICK1 allele, which is mapped in chromosome 20. Animals with the dominant allele exhibit a very short, sleek and glossy coat, enhancing thermoregulation in tropical environments, allowing cattle to maintain lower body temperatures and reduced HS. Prolactin is known to inhibit hair growth, and the slick phenotype seems to amplify this effect (Littlejohn et al., 2014). However, the exact mechanism by which the SLICK1 mutation alters prolactin signalling remains unclear. Probably, the SLICK1 mutation alters the prolactin signalling, leads to the suppression of hair growth, and thus resulting in the characteristic short and sleek hair coat (Mariasegaram et al., 2007). During episodes of HS, cattle with this coat type have lower body temperature, thanks to the enhanced conductive and convective ability and the lower absorption of solar radiation, and thus a better sweating rate (Dikmen et al., 2008). Although this gene was primarily identified in Senepol cattle, it was later introduced into Holstein cows. The slick gene has also been identified in different breeds, like Limousin, Holstein, Carora, Criollo and Romosinuano (Olson et al., 2003; Mariasegaram et al., 2007).

Overall, identifying climate-resilient animals on a genetic basis can be an important baseline to start breeding and conservation strategies aimed at developing and maintaining livestock populations that are well adapted to specific agro-climatic zones, combining thermotolerance, drought resistance, and the ability to thrive in low-input systems. Consequently, it is essential to evaluate each breed's adaptive traits under the climatic conditions to which they are, or will likely be, exposed (Sejian et al., 2018).

1.7. Sicilian local breeds

In Sicily, the largest Italian island hosts some important indigenous livestock breeds with unique physiological and genetic adaptations. These animals survive and reproduce under the harsh climatic conditions of this region. Because of its central position within the Mediterranean basin, the Sicilian climate is comparable to that of other Mediterranean regions such as Spain, Greece, Turkey, and North Africa, all characterised by hot, dry summers, with increasingly drought problems due to climate change.

Against this climatic background, livestock farming remains a key component of Sicily's regional economy. Recent statistics indicate that Sicily hosts a substantial cattle population estimated at 317.312 in total (Sistema Informativo Veterinario - Statistiche, 2025). This herd is composed of crossbreeds (194.580), followed by the following breeds in descending order Limousine: (44.595), Holstein (30.471), Charolais (14.980), Cinisara (7.517) and Modicana (6.389). The Cinisara and Modicana are two Sicilian cattle breeds exhibiting a strong capacity to the challenging conditions of Sicily's marginal mountainous areas, thanks to their good grazing behaviour and resilience to environmental conditions (Mastrangelo et al., 2014). Indeed, although the productivity of these two local breeds is lower when compared with the cosmopolitan cows, their performance remains more consistent during HS, or episodes of drought and feed scarcity. Moreover, these animals are kept on pasture throughout the year and are able to thrive on poor quality feeds.

The Cinisara breed (Figure 6) is found in different areas of Palermo province (mainly in the mountainous territory of Cinisi) and is well adapted to the harsh, hot and humid environmental conditions typical of this northwest area of Sicily. This breed is included in the "Registro Anagrafico delle razze bovine autoctone a limitata diffusione" under the conservation and protection of breeds with limited diffusion category (Maniaci et al., 2020).

The Cinisara breed is characterized by a very robust skeletal structure with a uniform black coat. Although it is a dual-purpose breed, the main attitude is milk production (about 3.700 kg of milk/lactation), which is used to produce "Caciocavallo Palermitano". This milk is rich in protein (3.5%) and fat (3.6%) content (Altomonte et al., 2016), but also in polyphenols and unsaturated fatty acids (Caracappa, 2023).

Figure 6. Cinisara cattle.

On the other hand, the Modicana breed (Figure 7) is reared in the province of Ragusa, in the south-western part of Sicily, a region having hot summers and a landscape characterised by hilly inland areas. The breed is characterized by a red coat with a robust constitution (which has allowed it to be historically used for draft work), and it was traditionally used for both milk and meat production. The Modicana cows produce around 4.000 kg of milk/lactation, which is mainly used for the production of Ragusano PDO cheese, which is important economic driver of the region. Similar to the Cinisara, the Modicana's milk is also rich in fat (3.78%) and protein (3.47%) (Ruminantia,

2022) This milk based on a diet consisting mainly of pasture, imparts the typical organoleptic flavour of the Ragusano PDO cheese. The Modicana together with Cinisara, is recognised as a Slow Food Presidium, highlighting their cultural relevance and conservation value.

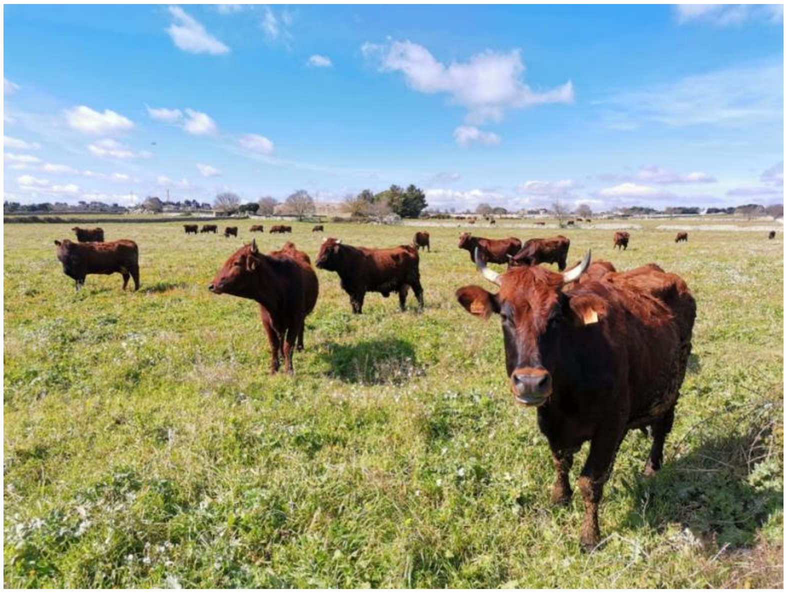

Figure 7. Modicana cattle

Indeed, both breeds play an important role not only in the regional economy but also in the cultural and historical tradition of Sicily. However, in recent years, they have largely been replaced by cosmopolitan breeds such as Holstein and Brown Swiss, placing them at risk of extinction. A key starting point for preventing their decline is to enhance their value by demonstrating their thermo-resilience, while at the same time assessing whether these breeds also experience HS in order to develop targeted mitigation strategies.

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CHAPTER 2

Aim of the Research

Based on the evidence that HS compromises health, welfare and performance in dairy cows, and from the concept that cattle breeds experience HS at breed-specifically THI, this thesis aims to investigate the thermotolerance in the Sicilian local breed, the Modicana and Cinisara cows, reared under semi-extensive condition. These breeds represent an important resource for the rural economy and the tradition of the region, whose maintenance could support biodiversity conservation and sustainable dairy farming in the Mediterranean area. The studies focused on the evaluation of key physiological parameters such as rectal temperature, oxidative stress, milk yield and quality, in order to objectively assess HS response compared with other cosmopolitan breeds that have already been widely studied.

The initial chapter (Chapter 3) on Cinisara cow described the thermoresistance of this breed through the relationship between THI and rectal temperature, milk production and quality. Results indicate that the Cinisara cows can tolerate high THI values before showing physiological and productive signs associated with HS, highlighting its natural adaptability to Mediterranean conditions and supporting the need to define breed-specific THI thresholds.

The next chapter considers the impact of HS on oxidant and antioxidant plasma markers of Modicana cow (Chapter 4). The findings indicate that high thermal load disrupts plasma redox balance by increasing oxidative metabolites and reducing antioxidant capacity. Nonetheless, the outcomes highlight that the antioxidant system of the resilient breed can also be impacted by HS. These results pointed out on the need to improve environmental management and target nutritional strategies in Sicilian dairy systems, to better support cows in coping with increasingly frequent thermal challenges.

Among the possible feeding strategies that can be adopted, the supplementation of rumen-protected dry grape extract, which is rich in polyphenols, was tested in Fleckvieh cows (Chapter 5), where it supported milk production, behavioural traits, and immunometabolic status during a heatwave. Indeed, the polyphenols content improved the overall thermotolerance capacity, suggesting its potential as a valuable dietary tool.

CHAPTER 3

Estimation of heat stress thresholds in Sicilian Cinisara lactating cows under naturally occurring conditions

SUMMARY

Cinisara cow responses to natural heat stress. The Cinisara cow is a native Sicilian breed adapted to local environmental conditions. However, few studies have investigated its effective resilience during heat stress conditions. This study aimed to evaluate how increasing temperature-humidity index (THI) during summer affects milk production, milk quality, and body temperature of this breed raised in semi-extensive system. The results demonstrated that Cinisara cows were able to tolerate higher THI levels compared to commercial breeds such as Holstein cows. Indeed, both rectal temperature and milk performance were affected when THI exceeded 77. Below this threshold, rectal temperature, milk quantity and quality were only slightly affected by THI. These findings suggest that Cinisara breed has an effective resilience to high temperature, likely due to its genetic background and historical management. Thus, supporting and preserving native breeds like the Cinisara could represent a strategy to reduce the impact of climate change on dairy farms.

Highlights

- Cinisara cows in pasture cope well with heat stress on milk yield and RT
- Milk yield and quality remained stable until THI peak of 77.
- RT and milk yield showed THI thresholds of 77.65 and 76.9, respectively
- The study supports the genetic potential of Cinisara cows' heat stress resilience

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Abstract

The efficiency of thermoregulation varies among breeds of cattle and can be part of the adaptive ability of local cows, such as Cinisara. This study aimed to assess the resilience to heat stress (HS) of Cinisara cows in a semi-extensive system during summer, evaluating rectal temperature (RT), milk yield (MY) and composition. The study was carried out from June to September 2024 on a commercial dairy farm located in Cinisi (Sicily, Italy) involving 15 primiparous and 20 multiparous lactating Cinisara cows. Temperature-humidity index (THI) was calculated based on temperature and humidity data registered by 3 probes every 15 min. The RT was recorded once a month after the morning and evening milking. The MY was recorded once a month, and milk samples were collected at that time. Data were analyzed with the PROC GLIMMIX and NLIN of SAS. Parity did not affect both RT and milk performance response to HS. Overall, the highest RT was registered in August in both morning and afternoon, when THI was above 77 (38.51 and 39.21°C, respectively). The nonlinear regression (NLIN) analysis revealed a strong and statistically significant relationship between the THI and RT (adjusted $R^2 = 0.89$). A THI breakpoint of 77.65 was identified for RT, beyond which RT increased significantly and abruptly by approximately +0.12 °C for each unit increase in THI above the threshold. The nonlinear regression analysis applied to milk yield data revealed a strong relationship between THI and milk production (adjusted $R^2 = 0.90$). A THI breakpoint of 76.9 was identified, beyond which milk yield declined sharply by approximately –8.77 kg/d for each unit increase in THI above the threshold, compared with a much milder decline (–0.69 kg/d for each unit) observed below the breakpoint. This highlights the breed's notable heat tolerance and its suitability for semi-extensive dairy systems in hot climates. Further studies exploring the genetic basis of this adaptation may support conservation and enhancement strategies for native breeds in Mediterranean regions.

Key words: heat stress; cows; local breeds; thermoregulation.

The pressure of selection for increase milk production is making cows more susceptible to HS since the increase of productivity results in increased metabolic heat production (Bernabucci et al., 2010). Moreover, the increase of endogenous heat production is caused by the request to consume more feed, and thus from nutrient digestion (Nasr and El-Tarabany, 2017). However, most studies investigating the effects of HS on dairy cows have focused on Holstein dairy cows (Wheelock et al., 2010; Ouellet et al., 2021), aiming to identify out the negative impacts on production, physiology, reproduction, and immunity, as well as evaluating the THI threshold for this breed, which is the most widespread in the world. Nevertheless, previous studies have evaluated differences in THI threshold in Brown Swiss cattle, showing higher thermal tolerance compared with Holstein cows, observing a greater impact on protein yield between the two breeds (Maggiolino et al., 2020a). These differences are more evident for local breeds, as their production potential is less pronounced and as they are adapted to live in harsh environment, due to their genetic and physiological adaptation (Rojas-Downing et al., 2017). In the Sicilian context, Cinisara cow is important for local economy and for its adaptation to harsh environment and climate, living in marginal pasture and being efficient in terms of milk quality, even with low-quality feed (Liotta et al., 2011). The Cinisara cow is a native Sicilian cow that can make productive use of the natural pastures of the hilly and semi-arid areas of the Sicilian Island. The Cinisara is a medium-sized cattle breed belonging to the group of Podolica cattle. It is characterized by a robust skeletal structure and is recognized as a distinct local Sicilian ecotype. The breed derives its name from the town of Cinisi, a coastal locality in western Palermo. Morphologically, the Cinisara is distinguished by its black coat, which is often uniform. The Cinisara plays an important role in the conservation of genetic diversity among Sicilian livestock and is valued for its milk production, used in the making of traditional cheeses. In fact, Cinisara cow is historically linked to the production of Caciocavallo Palermitano. The cheesemaking production of Caciocavallo Palermitano involves the use of whole and raw cow milk. Considering that Cinisara cow has a low level of management without cooling system, and generally raised under an extensive system, one of the most important factors limiting its production is heat stress (HS), especially for the resulting economic losses (Tao et al., 2020). Actually, there are no studies assessing HS response in local cattle breeds such as the Cinisara cow. Summer in Sicily is characterized by a hot-summer Mediterranean climate, with meteorological conditions driven by subtropical high-pressure systems and warm air masses from North Africa. During June, July, and August, average daytime temperatures range between 28°C and 35°C, though heatwaves can push maxima above 40 °C, particularly in inland valleys. Several authors aimed to point out that local breed cows over time have adapted to specific environments, soil, and climate conditions (Ciotola et al., 2009; Di Gregorio et al., 2017), defining local breeds as "rustic or resistant" compared with world-wide cows such as the Holsteins. But what contributes to the rusticity or resistance of these local cows? We hypothesize that Cinisara cows could have a high critical THI threshold at which they exhibit physiological signs of heat stress. Thus, our objective was to establish the THI breakpoint for RT of lactating Cinisara cows and evaluating the response of milk production and its composition over a 90-d period of naturally occurring heat stress during summer.

The protocol for animal handling and care was reviewed and approved by the University of Messina Animal Care and Use Committee under application number 19/2023. The study was carried out between June and September in a commercial dairy farm located in Cinisi (Sicily, Italy). A total of 35 lactating Cinisara cows, 15 primiparous and 20 multiparous, (DIM: 114 ± 32 d; milk yield: 20.05 ± 2.43 kg/d) were enrolled and monitored over a 90-d period during natural heat stress exposure in the summer. All cows were raised in a semi-extensive dairy system, grazing most of the time (approximately 18 h/d) and housed in the barn only for milking and feeding (twice a daily). During milking, all cows were individually offered 3 kg of concentrate per head (on DM basis: 17.4 % of CP, 5.5 % of EE, 45.4 % of starch, and 6.87 % of ash) delivered at 07:00 and 17:00. Before being sent out for grazing, each cow was given approximately 3 kg of grass hay (on DM basis: 10.4 % of CP, 56.8 % of NDF, 33.5 % of ADF, and 6.2 % of ADL). Ambient temperature and relative humidity to calculate the Temperature-Humidity Index (THI) were recorded every 15 minutes using three thermo-hygrometers installed at different locations within the grazing area (Govee IT, model: part_9894). The THI was calculated based on the equation of Thom (1959): $\mathrm{THI} = (1.8 \times T + 32) - [(0.55 - 0.0055 \times RH) \times (1.8 \times T - 26)]$, where T = ambient temperature (°C) and RH = relative humidity (%).This equation was chosen because it is one of the most widely used in animal science literature, allowing for easier comparison with previous studies. The daily mean, minimum and maximum values of THI recorded during the study period are shown in the Figure 1A.

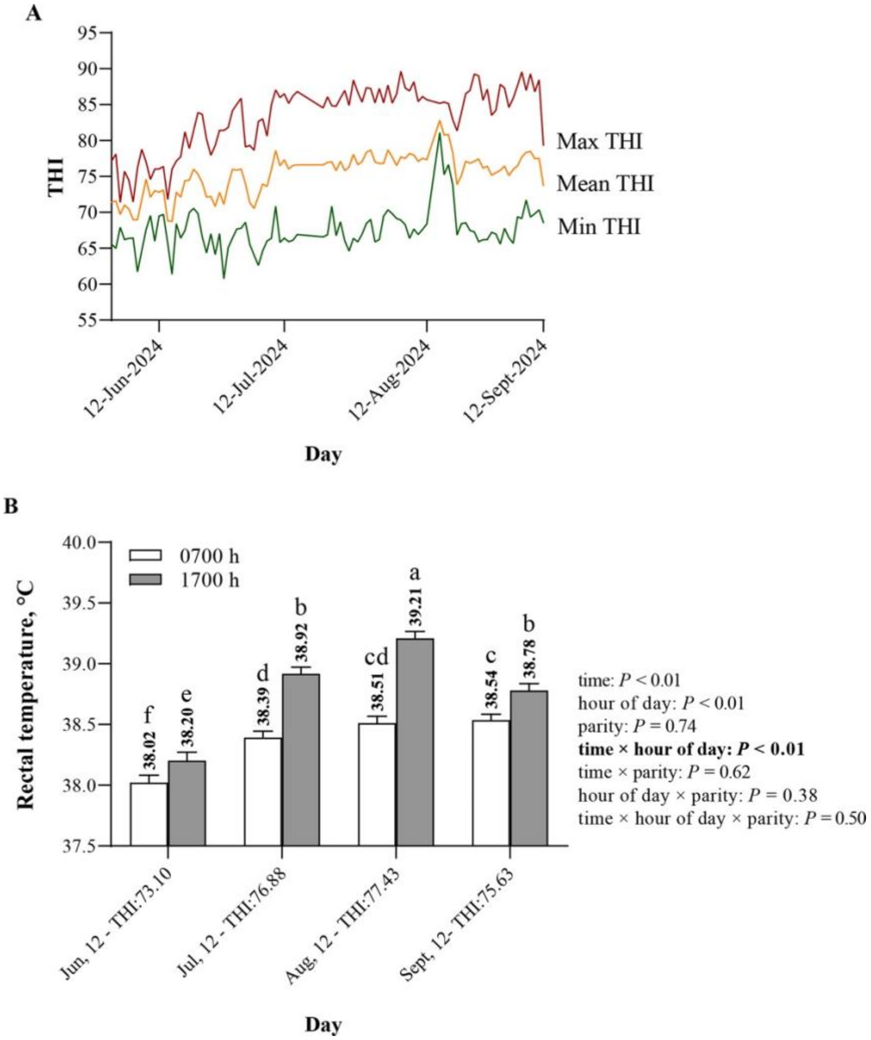

Figure 1. Trend of THI and LSM ± SE of morning (07:00 h) and evening (17:00 h) rectal temperature (°C) of Cinisara lactating cows over a 90-d period during natural heat stress exposure in the summer. Bars represent the standard error of the mean and different letters indicate statistically significant differences ($P \leq 0.05$).

The average THI values at each time point were calculated through the arithmetic mean of mean THI of 4 consecutive days (the day of measurements and samples collection and 3 d before). Rectal temperature (RT, °C) was monthly recorded after the morning (07:00h) and evening milking (17:00h). The RT was measured using a clinical digital thermometer (TFA Dostmann Thermometer VET 112, Germany) inserted into the rectum. Milk yield (MY) was recorded, and milk samples were collected in June, July, and September within the Test-Day Measures. However, milk samples were not collected in August due to a sudden machine breakdown. Milk samples were analyzed for fat, protein, lactose, casein, urea, total milk solids, non-fat milk solids, citric acid, fatty acids, BHB by mid-infrared spectroscopy (Milkoscan FT2, Foss Electric, Hillerød, Denmark), and somatic cell count (Fossomatic, Foss Electric).

Data were analyzed using SAS software (version 9.4; SAS Institute). Normality of data was checked by using the univariate procedure of SAS. Milk data were analyzed as repeated measurements with the GLIMMIX procedure of SAS. The statistical models included the fixed effects of time (Jun, Jul, Aug and Sept), parity (primiparous and multiparous), and their interaction, whereas individual cows were included as random. The model equation was: $Yijk = \mu + Ti + Pj + TPij + ck + \varepsilon ijk$, where $Yijk$= dependent continuous variable, μ = overall mean, Ti = fixed effect of time, Pj = fixed effect of parity, $TPij$ = interaction between time and parity, ck = random effect of mth animal (cow), and εijk = residual error. Rectal temperature data were analyzed as repeated measurements with the GLIMMIX procedure of SAS. The statistical models included the fixed effects of time (Jun, Jul, Aug and Sept), hour of day (07:00h and 17:00h), parity (primiparous and multiparous), and their interaction, whereas individual cows were included as random. The model equation was: $Yijkl = \mu + Ti + Hj + Pk + THij + TPik + HPjk + THPijk + cl + \varepsilon ijkl$, where $Yijk$= dependent continuous variable, μ = overall mean, Ti = fixed effect of time, Hj = fixed effect of hour of day, Pk = fixed effect of parity, $THij$ = interaction between time and hour of day, $TPjk$ = interaction between time and parity, $HPjk$ = interaction between hour of day and parity, $THPijk$ = interaction between time, hour of day, and parity, cl = random effect of mth animal (cow), and $\varepsilon ijkl$ = residual error. Comparisons with $P \leq 0.05$ were considered significant. Finally, regressions were performed between the least square means of rectal temperature data retrieved from the mixed models and THI values. In detail, segmented regression analysis was conducted using the NLIN procedure to identify the THI breakpoint at which RT began to increase significantly ($P < 0.05$) and abruptly considering all cows.

In order to assess heat tolerance in dairy cows, the monitoring of RT and respiration rate are commonly used (Bernabucci et al., 2010) as first indicators for heat stress detection. Dairy cows maintain the body temperature balancing the heat gain and the heat loss (Hahn, 1999). When skin surface temperature exceed 35°C, cows begin accumulating heat and RT increases (Bakony et al., 2023), causing changes to the physiology of the animal. For this reason, during HS when THI is above 70, the RT increases beyond 39°C (Yan et al., 2021).

In this study, although parity and its interaction with time and/or hour of the day did not affect RT ($P > 0.10$), a significant increase of RT was observed during the afternoon hours compared to the morning hours ($P < 0.05$), as shown in Figure 1B. During the morning hours, the highest overall RT was registered in August (38.51 ± 0.06°C; THI = 77.43) and September (38.54 ± 0.05°C; THI = 75.63), indicating a direct association between RT and THI, whereas the lowest RT was registered in June (38.02 ± 0.06°C; THI = 73.10). Moreover, the highest RT registered during the afternoon hours was in August (39.21 ± 0.06°C), where the THI reached 77.48, whereas the lowest RT was registered in June (38.20 ± 0.07°C) with a THI of 73.10 (Figure 1B). These results showed that RT was consistently higher in the afternoon hours (17:00 h) compared to the morning hours (07:00 h). This diurnal variation in dairy cows was previously reported and reflects the circadian rhythm of the animal, with a peak in the afternoon and a minimum in the morning, also as a consequence of environmental heat load throughout the day (Theurer et al., 2014).

Moreover, this diurnal fluctuation was relatively small (0.7°C daily fluctuation in August), compared to that reported by Islam et al. (2023) for both heat-susceptible and heat-tolerant feedlot beef heifers in Australia, indicating that heat-susceptible heifers had a 1.5 °C daily RT fluctuation (38.24°C to 39.76°C) and heat-tolerant cattle had a 0.87 °C daily RT fluctuation (38.13°C to 39.00°C).

Temperature-humidity index threshold was determined when abrupt and significant changes in the rectal temperature (RT) were detected at certain THI value (Figure 2A). A strong correlation (adjusted R2 = 0.89) was observed between THI and RT, and a THI threshold was identified for RT. Cinisara cows exposed to natural HS exhibited a THI breakpoint of 77.65 for RT, whereby RT began rising at a rate of 0.04 °C for every unit increase in THI below the threshold (Figure 2A), whereas RT began rising at a rate of 0.12 °C for every unit increase in THI above the threshold (Figure 2A). Several studies show that Holstein and Brown Swiss dairy cows exhibit a THI threshold between 70 and 75 for a RT of 38.5 °C, depending on physiological stage and parity. (Maggiolino et al., 2020b; Pinto et al., 2020; Yan et al., 2021). In contrast, Cinisara cows demonstrate a higher THI threshold and a slower increase in RT as THI rises. Previous studies on indigenous local breed, such as Bos Indicus, suggested that skin structure could explain the better thermoregulation properties, thanks to sweat gland structure (Mateescu et al., 2023) and skin thickness, facilitating the increased blood flow to the skin during HS (Carvalho et al., 1995). Thus, can be assumed that Cinisara cow is better able to regulate body temperature in response to HS compared to Holstein and Brown cows, probably being able to maintain the balance between the ability to dissipate heat through skin and the lower metabolic rate. This is also demonstrated by the maintaining of RT in the physiological range in both morning and evening hours, without occurring in hyperthermia even during the summer months. However, further studies on the structural characteristics of the skin and hair of Cinisara cows could help to clarify their enhanced heat dissipation mechanisms.

Temperature-humidity index threshold was determined also for milk yield (Figure 2B). Despite the limited number of time points (n = 4), segmented linear regression applied to average daily milk production data identified a breakpoint at THI =76.9 (Figure 2B). Before the threshold, the decline in milk yield was moderate (–0.69 kg/d per THI unit; 95% CI: –1.14 to –0.23 kg/d), while beyond this point, milk production dropped significantly (–8.77 kg/d per THI unit; 95% CI: –11.84 to –5.71 kg/d). The difference in slope (Δ = –8.09 kg/d; 95% CI: –11.58 to –4.59 kg/d) reflects a marked reduction in milk output at high THI levels (Figure 2B). The model showed a strong correlation (adjusted $R^2 = 0.90$).

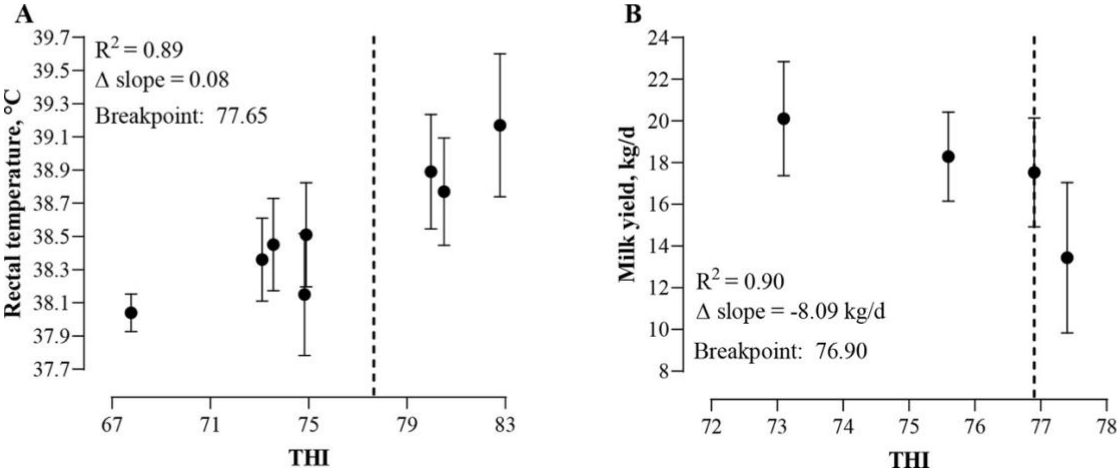

Figure 2. Nonlinear segmented regressions of rectal temperature (°C, LSM ± SD; A) and milk yield (kg/d, LSM ± SD; B) in lactating Cinisara cows relative to THI over a 90-d period during natural heat stress exposure in the summer. Vertical line indicate breakpoint at which rectal temperature and daily milk yield changed significantly and abruptly. Δ slope represent the change in slope between the slope of data before breakpoint (b1) and the slope of data after breakpoint (b2).

Regarding the milk production and its composition response, parity and its interaction with time did not affect milk data (P > 0.10). Overall, milk yield (MY) was significantly affected by time (Time; P < 0.01), reflecting the trend of THI, during the 90-d of the monitoring period (Figure 3A). Specifically, MY was on average 20.11 ± 0.70 kg/d in June (THI = 73.10), decreased to 17.53 ± 0.57 kg/d in July (THI = 76.88), reached its lowest value of 13.43 ± 0.5 kg/d with the highest THI in August (THI = 77.43), and then increased in September to 18.29 ± 0.62 kg/d when THI reached lower levels (THI = 75.63).

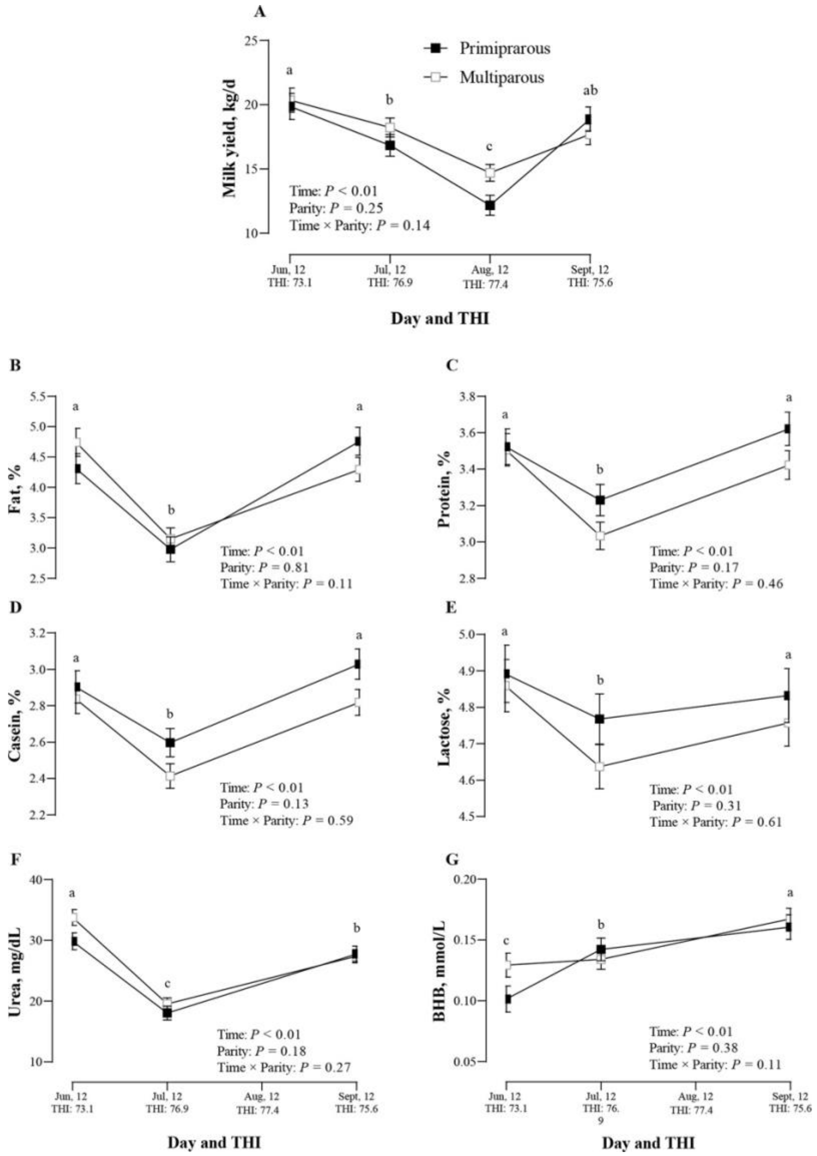

Figure 3. Milk yield and milk composition (LSM ± SE) in Cinisara lactating cows over a 90-d period during natural heat stress exposure in the summer. Bars represent the standard error of the mean and different letters indicate statistically significant differences among time points ($P \leq 0.05$).

Previous studies on Holstein cows, defined the threshold for milk production around 68, where cows started to significantly reduce the MY, with further decline at higher THI values (74 or 76) (Bernabucci et al., 2014; Heinicke et al., 2018). However, several studies have evaluated a different MY response among breeds during HS. For example, Collier et al. (1981) suggested that Jersey cows are more heat tolerant than Holstein cows in relation to milk production, and Smith et al. (2013) observed an increase in milk production in Jersey and a decrease in Holstein cows under HS. Our recent study revealed that mid-lactating Fleckvieh cows showed a THI threshold for milk yield around 73, where cows started to significantly reduce the MY at a rate of approximately 1.5 kg/d at THI above 73 (Amato et al., 2025). This result suggests that this local breed may have developed mechanism to better tolerate high temperature maintaining the milk production until higher THI (around 77), and thus a better resistance to HS. While a reduction of approximately 8 kg/day in milk yield beyond the THI breakpoint may appear excessive, it is important to note that this drop occurs only after exposure to a particularly high THI. In comparison, most conventional dairy breeds begin to show declines in milk production at THI values between 69 and 73. The Cinisara breed, by contrast, is able to maintain stable milk yield up to a THI of 77 (even though Cinisara is a low-producing breed). Indeed, below the threshold, milk yield decreased only marginally, with a slope of –0.69 kg/d per unit of THI (95% CI: –1.14 to –0.23 kg/d), suggesting a higher tolerance to heat stress. Thus, although the overall reduction is similar compared with other milk-specialized breeds, the Cinisara appears to delay the negative effects of heat, highlighting its adaptive advantage under hot environmental conditions. However, it is noteworthy to consider that overall, the MY of Cinisara cows is lower compared with Holstein cows (on average 3,700 kg of milk per lactation vs. 10,000 kg, respectively); thus, the lower MY and therefore the lower metabolic heat, contribute to reduced susceptibility to HS. Environmentally induced hyperthermia not only affects overall milk production but also milk composition, decreasing fat, protein and lactose content (Mylostyvyi and Chernenko, 2019; Florio et al., 2022).

Consistently with this, results on Cinisara milk quality (Figures 3B-G), exhibited significant variations (Time; $P < 0.01$) over time and THI, with a decrease in milk components during the hottest period of the trial in July, when THI was above 76. In fact, in July (THI = 76.88) a decrease in milk fat (Jun: 4.53 ± 0.17 %; Jul: 3.06 ± 0.14 %; Sep: 4.52 ± 0.15 %), protein (Jun: 3.51± 0.07 %; Jul: 3.13 ± 0.06 %; Sep: 3.52 ± 0.06 %), casein (Jun: 2.87 ± 0.06 %; Jul: 2.50 ± 0.05 %; Sep: 2.92 ± 0.06 %), lactose (Jun: 4.88 ± 0.05 %; Jul: 4.70 ± 0.05 %; Sep: 4.79 ± 0.05 %) and urea (Jun: 31.81 ± 0.96 %; Jul: 18.80 ± 0.76 %; Sep: 27.59 ± 0.85 %) content was observed compared with June (THI = 73.10) and September (THI = 75.63), where their levels were higher. The decrease in milk fat is mainly related to the decrease in DMI (Collier et al., 2006), which leads to the decrease of ruminal production of acetate, a precursor for milk fat (Kim et al., 2022). However, it is noteworthy that after the peak of THI, the milk fat content of Cinisara cows reached greater levels. Similarly, the decrease in milk protein and urea during the peak of THI could be related to the decrease in DMI, but this mechanism can be compromised also by the alteration in amino acid circulation (Gao et al., 2017), as amino acids are redirected to support maintenance of metabolism than for milk synthesis (Cartwright et al., 2023). Indeed, Cowley et al. (2015) pointed out that these reductions in milk protein of heat-stressed cows appear to be a result of specific downregulation of mammary protein synthetic activity, exacerbated by the increased protein turnover, AA competition between casein and structural proteins. In addition to mammary gland intrinsic mechanisms, heat stress-induced milk protein reductions might be the result of limitations in the precursor supply caused by the reduction in mammary blood flow. When dairy cows rely predominantly on pasture, long periods of dry weather during summer and HS can reduce both overall biomass intake and nitrogen intake from forage. This alteration significantly impacts ruminal protein metabolism, resulting in a decreased supply of precursors for milk protein synthesis due to a reduction in rumen microbial protein production (Bernabucci et al., 2015). This decline in microbial protein synthesis may partly explain the observed reduction in milk urea levels during the hottest periods. In contrast, previous studies involving cows fed total mixed rations have reported increased blood and milk urea levels under induced and naturally occurring heat stress conditions (Bernabucci et al., 2014; Gao et al., 2017; Hou et al., 2021; Maggiolino et al., 2025), likely originating from both inefficient rumen ammonia incorporation into microbial protein and hepatic deamination of AA mobilized from skeletal muscle (Bernabucci et al., 2015). Thus, we speculate that the urea metabolism of Cinisara cows raised in an extensive system, where pasture is the primary nutritional source, is more susceptible to nutritional influences than to the direct effects of heat stress.

A similar pattern of metabolic adaptation was observed in energy metabolism, as evidenced by changes in BHB levels. Indeed, BHB content was conversely lower in June (Jun: 0.12 ± 0.01 mmol/L), and then significantly increased during the peak of THI in July (Jul: 0.14 ± 0.01 mmol/L) until reaching its highest levels in September (Sep: 0.16 ± 0.01 mmol/L). A previous study also observed rise in BHB in cows which experienced HS, reflecting fat mobilization for reducing energy intake (Ishida et al., 2024). This study observed that during HS milk BHB increased of 0.1-0.3 mmol/L, compared to cooled cows, reflecting our result. However, the significant increase of BHB during the month of September can be related to the increase in MY, which raises the energy requirements of the cows. However, the role of pasture cannot be excluded. Indeed, given that Cinisara cows depend primarily on natural pasture for their nutritional intake, the observed decline in milk yield and its composition during the hotter periods (July and August) could be attributed not only to the direct effects of heat stress, but also to a significant reduction in both the biomass and the energy content of the available forage. As temperatures rise and drought conditions intensify, the quality of the pasture tends to deteriorate, with lower levels of digestible nutrients and energy, thereby compromising the animals' ability to meet their physiological requirements for sustained milk production. These results suggest that Cinisara cow exhibit good tolerance to HS, maintaining RT within physiological range even during the peak of summer. Although milk production and quality were negatively affected by HS, they started to being triggered at higher THI levels. Moreover, the impact was less pronounced compared to those observed in Holstein and Brown cows in previous studies. Thus, the better resilience and adaptability to harsh environmental condition of Cinisara cow can be a resource for the future of dairy farming in the Mediterranean area. Further studies are needed to explore the genetic and physiological mechanisms involved in the heat tolerance of Cinisara cattle, to preserve and promote biodiversity conservation.

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CHAPTER 4

Impact of Heat Stress on the Balance between Oxidative Markers and the Antioxidant Defence System in the Plasma of Mid-Lactating Modicana Dairy Cows

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Simple Summary: This study was conducted to determine the effects of the incremental temperature humidity index (THI) on oxidant and antioxidant plasma markers in mid-lactating dairy cows. Results showed a significant increase in oxidative markers and a significant decrease in antioxidant defence; this balance results in oxidative stress. Heat stress significantly modulated fat milk content, blood cell number, and plasma metabolite concentration.

4.1 Abstract

Animal health is affected during heat stress as a result of impaired immune responses, increased production of reactive oxygen species, and/or a deficiency of antioxidants. This leads to an imbalance between oxidants and antioxidants and results in oxidative stress. Heat stress is usually measured in dairy cattle via the temperature-humidity index (THI). In the present study, we aimed at assessing the influence of incremental THI on the balance between oxidative markers and the antioxidant defence system in the plasma of Modicana cows. Twenty-four multiparous, mid-lactating dairy cows were divided into two groups on the basis of different levels of mean THI reached in the period of the previous week up until the day of blood and milk sampling (April THI_1:55, May THI_2:68, June THI_3:71, July THI_4:80). The blood samples were collected to measure reactive oxygen metabolites (ROM) and advanced oxidation protein products (AOPP) on the one hand, and antioxidant defense markers (ferric reducing ability of plasma (FRAP), paraoxonase (PON), plasma thiol groups (SHp)), as well as lipid-soluble antioxidant pro-vitamin (β-carotene) and vitamins (tocopherol and retinol) on the other hand. Milk characteristics, haematological values, and plasma biochemical metabolites were also evaluated. Results showed a significant increase in ROM

($p < 0.05$) and a significant decrease in PON ($p < 0.05$), AOPP ($p < 0.05$), and β-carotene ($p < 0.001$). Incremental THI significantly decreased levels of milk fat content, red and white blood cells, plasma glucose, and non-esterified fatty acids, while significantly increasing monocytes and the concentrations of β-hydroxybutyrate and creatinine, but not fructosamine. The results of the study show that heat stress significantly affects reactive oxygen species production and antioxidant parameters. Carotenoid supplementation should be considered to alleviate the impact of these effects.

Keywords: THI; milk; reactive oxygen metabolites; antioxidant vitamins; plasma metabolites; dairy cows

4.2. Introduction

Livestock health and productivity are particularly susceptible to the negative effects of rising temperatures, especially in cases of drought. Although nowadays science is studying to adopt several strategies to improve the environmental condition and the resilience of the animals with the use of management strategies (e.g., sprinklers, fans), nutritional strategies, and innovative systems of watering, it is well demonstrated how genetics plays a fundamental role.

Heat stress reduces dry matter intake, negatively affecting milk yield and composition, nutrient absorption, and, as a consequence, impacting energy metabolism, the immune system, and the inflammatory response (Yadav et al., 2016). In the near future, animal life will be coping with heat waves that have increased intensity and last for longer than even the most devastating heat wave experienced to date (Stillman, 2019). Jacobs et al. (2020) used a heat wave simulator, where mice were first maintained at control temperatures for five days, then transitioned to temperatures that oscillated between a minimum of 22 ◦C and a maximum of 34 ◦C for one day, followed by heat wave temperatures which oscillated between a minimum of 24 ◦C and a maximum of 39 ◦C for a period of three full days. Heat wave exposure caused oxidative stress in different organs, which may have had negative consequences for animal physiology and life expectancy. Similar temperature conditions to those in the previous study occurred naturally in Sicily; in particular, the ambient temperature reached 50°C in the second half of July 2023.

Dairy cattle are more susceptible to heat stress compared to other livestock species. This susceptibility is due to breeding selections aimed at boosting milk production, which in turn increases metabolic heat output (Kadzere et al., 2002). Dairy cows with high levels of productivity, begin to lose the ability to regulate their body temperature when ambient temperatures surpass 30 ◦C (Meneses et al., 2021). Heat stress is usually measured in dairy cattle via the temperature-humidity index (THI). THI presents the combination of ambient temperature and relative humidity and is a useful and easy way to assess the risk of heat stress (H.H. Kibler, 1964).

A THI below 70 is generally considered to be comfortable, between 75 and 78 stressful, and above 78 hazardous (Kadzere et al., 2002). Above this threshold, cattle try to maintain body temperature by dissipating heat and undertaking numerous behavioral and physiological changes. These changes negatively impact the production, reproduction, and health of animals, in turn leading to economic losses for the farm. Specifically, the cow's immune system is strictly compromised since heat stress decreases the activity and the number of several neutrophils and lymphocytes (Tejaswi et al., 2020). Furthermore, high temperatures can induce oxidative stress, a condition resulting from an imbalance between the production of reactive oxygen species (ROS) and antioxidants.

Normally, antioxidants can neutralize excessive ROS to maintain redox balance (De Almeida et al., 2022). Heat-induced disruption of this balance leads to an increase in reactive oxygen metabolites (ROM), which damage cells and tissues and impair immune function (Belhadj Slimen et al., 2014; Giannone et al., 2023). Unlike ROS, ROM is more stable and can be easily quantified (Alberti et al., 2000). Additionally, the compromise of the immune system may result in an increased susceptibility of cows to several diseases. Along with ROM, various blood biomarkers are commonly measured and studied to assess the condition of oxidative stress in cows. Ferric Reducing Ability of Plasma (FRAP) measures the capacity of plasma to reduce Fe3+ (ferric ion) to Fe2+ (ferrous ion) based on the presence of available reducing agents. FRAP offers a putative index of the antioxidant, or reductive, potential of plasma during oxidative stress (Benzie and Strain, 1996).

Paraoxonase (PON) is an antioxidant enzyme whose activity is decreased in certain conditions associated with oxidative stress (Turk et al., 2015). New compounds that can serve as biomark- ers have been identified. Among these are advanced oxidation protein products (AOPPs), which are primarily formed by chlorinated oxidants produced through the activity of myeloperoxidase (Cristani et al., 2016). However, these AOPPs have not yet been evaluated during thermal stress in dairy cows. Specific protein cysteine thiols (SHp) have emerged as markers of redox status levels (Baba and Bhatnagar, 2018). Evaluating the levels of SHp could be interesting in understanding the response to thermal stress in dairy cows.

Carotenoids are a fascinating group of natural pigments. Not only are they responsible for a broad array of coloration in nature, but, more importantly, they have key functional roles in biology (Di Martino et al., 2018). They are precursors of vitamin A and present a robust antioxidant capacity that contributes to protecting the body against the effects of ROS (Di Martino et al., 2018). Similar to carotenoids, vitamin E is an essential antioxidant (Ralla et al., 2024).

A better understanding of the physiological variation in lactating dairy cows during extreme environmental conditions can help to improve their welfare and prevent thermal stress-related economic loss. The immune system is the body's primary defense mechanism for protecting against and coping with environmental stressors. White blood cells (WBCs), red blood cells (RBCs), hemoglobin (Hb), packed cell volume (PCV), glucose, and protein concentration in the blood are usually affected by thermal stress (Das et al., 2016). Another potential biomarker of stress is fructosamine, a glycated protein, as it reflects long-term blood glucose concentration (Grelet et al., 2022).

Within Italian autochthonous breeds, the Modicana is the most important native bovine breed in Sicily, both in terms of consistency and zootechnical quality. In particular, Modicana's milk is used to produce a cheese with a protected designation of origin (PDO) label, named "Ragusano", which is aged from 4 to 12 months. Modicana cows are usually reared in extensive systems, using pasture during the grazing season and with limited or no supplementation of concentrate to their diet. Semi-intensive farming practices are also possible when animals cannot go to pasture and need higher concentrate supplementation (Valenti et al., 2019). Modicana cows are appreciated by breeders for their maternal behaviors and for their adaptability to adverse conditions, dietary in particular, and their milk has higher levels of biomolecules and antioxidant activity compared to Holstein (Salzano et al., 2022). Due to the rusticity of this breed and the hypothesis of its greater resilience to adverse weather conditions, the objective of this study was to investigate the effects of thermal stress on the balance between oxidative markers and antioxidant defense in the plasma of Modicana cows.

4.3. Materials and Methods

4.3.1. Animal Management and Treatment

The present study was performed in a commercial dairy farm with the Modicana breed located in the province of Ragusa, Sicily, Italy (36◦56′49″ N 14◦41′50″ E, 500 mt above sea level) under the traditional semi-intensive farming practice. This dairy cattle farm was monitored from April to July 2022. The study was approved by the Ethics Committee of the Department of Veterinary Sciences, University of Messina, code number 041/2020. A total of 24 healthy lactating Modicana dairy cows [Body Condition Score (BCS): 2.75 ± 0.15; parity: 3.17 ± 1.49; days in milk (DIM): 158 ± 37 d; and milk yield (MY): 11.38 ± 3.7 kg/d (mean ± SD)] were selected: two groups each for evaluation on two different environmental conditions of THI (Group 1 April, May, Group 2 June, and July). The cows in this study were examined daily for health-related problems via visual observation, a temperature check, and monitoring milk yield by trained personnel (see Table 1 for individual characteristics).

Table 1. Names, number of lactations, and days in lactation (DIM) of Modicana dairy cows involved in the study.

Group 1	Number of Lactations	DIM at T1	Group 2	Number of Lactations	DIM at T3
Fortunata	4	147	Ardua	4	126
Gioia	3	137	Effe	3	151
Ibla	2	152	Elisea	3	150
Iblea	2	122	Elvana	3	111
Iena	2	148	Formia	5	115
Imperia	2	133	Irianna	2	144
Messina	3	84	Lisa	2	127
Angela	6	110	Luisa	2	146
Batia	5	112	Melinda	2	217
Delia	5	110	Michelle	2	146
Elisa	3	180	Nina	2	123
Zeta	4	110	Siciliana	5	108
Mean ± s.d.	3.42 ± 1.38	129 ± 26		2.91 ± 1.16	139 ± 29

Cows were fed hay ad libitum and concentrate according to their milk production (on average 8.7

kg/head/day as dry matter of concentrate in two equal meals in the morning and afternoon during the milking). Feed formula and nutrients are reported in Table 2. The cows were milked twice daily (6 a.m. and 6 p.m.). After each milking, cows were allowed to pasture for the entire period under investigation (for a minimum of 6 h during daylight, from 8:00 a.m. to 2:00 p.m.). The animals had free access to water, both indoors and outdoors (the indoor housing was a free-stall barn). The animals were grouped based on body condition score (BCS) and parity and then assigned to two groups to ensure similar days in milk and milk yields (n = 12 animals per group). The selected animals were multiparous, mildly lactating dairy cows and were evaluated for blood and milk parameters across four periods.

Table 2. Ingredients and chemical composition of the diet of Modicana cows.

Ingredients, % of DM	
Corn meal	40
Roasted soybean flour	16.5
Barley meal	12
Beetpulp	9
Wheat bran	6
Sunflower meal	6
RUMEN Bypass Fat	2.5
Minerals and Vitamins Mix	1.5
Calcium Carbonate	1.3
Saccharomyces dried yeast	1
Cane molasses	1
Na bicarbonate	1
Na chloride	0.7
P dicalcium	0.6
NutriGen 40 C	0.5
Mg oxide	0.4
Chemical composition, % of DM	
Crude Protein	17.7
Fat	5.01
Starch	45.64
Crude Fibre	4.40
Ash	9.84
Nel, Mcal/kg of DM	1.81

4.3.2. Environmental Data

Climate data used for this study was obtained on an hourly basis from a weather station installed in Ragusa (36◦56′ N, 14◦44′ E). The mean temperature (T) and relative humidity (RH) recorded from the previous week until the day of sampling (T1 = 11 April, T2 = 27 May, T3 = 17 June, and T4 = 26 July) were used to calculate the related temperature humidity index (THI) using a formula previously described (National Research Council, 1971):

$THI = (1.8 \times T + 32) - [(0.55 - 0.0055 \times RH) \times (1.8 \times T - 26.8)]$

where T is the air temperature (◦C) and RH is the relative humidity (%).

4.3.3. Milk Performance and Analysis

Consecutive evening and morning milk samples were individually collected monthly (April, May, June, and July) with a manual milk sampler (Waikato Milk Metre, Milkline Company, Piacenza) and composited in proportion to milk yield. Milk yield was recorded, and individual milk samples (60 mL) were collected to assess milk quality (fat, protein, casein, and lactose) using a Fourier transform infrared analyzer (Milkoscan FT2, FOSS, Hillerød, Denmark).

4.3.4. Blood Sample Collection and Analysis

For each cow, blood samples were collected monthly before the morning feed from the jugular vein using an 18-gauge Vacutest Kima needle (Vacutest Kima SRL, Arzergrande, Italy). The samples were drawn into two types of tubes: 9-mL evacuated test tubes con- taining lithium heparin and 6-mL K-EDTA tubes (Vacumed, Padova, Italy). The collected samples were immediately cooled in an ice-water bath. After collection, the blood samples were collected into lithium heparin tubes centrifuged at 1900× g for 16 min at 4 ◦C. The resultant plasma was aliquoted and stored at −20 ◦C until further analysis.

Plasma metabolites were analysed at 37 ◦C by using an automated clinical analyzer (ILAB 650, Instrumentation Laboratory Company, Lexington, MA, USA), as reported by Lopreiato et al. (2021). Commercial kits were used to measure glucose, total cholesterol, urea, calcium, in- organic phosphorus, total protein, albumin, total bilirubin, and creatinine (Instrumentation Laboratory SpA, Milan, Italy), nonesterified (free) fatty acids (FFA), and β-hydroxybutyrate (BHB) (kit Ranbut, Randox Laboratories Ltd., Crumlin, UK). The total globulin fraction was determined by subtracting albumin from the total protein. The ratio of albumin to globulin A/G was then calculated. ROM was measured as described by Bionaz et al. (2007). Plasma paraoxonase (PON, EC 3.1.8.1) activity was assessed by adapting the method of Ferre et al. (2002) to the ILAB 650. Ferric-reducing antioxidant power (FRAP) was measured using the colorimetric method of Benzie and Strain (1996) and advanced oxidation protein products (AOPP) as described by Hanasand et al. (2012). Plasma thiol groups (SHp) were determined by titration with 5,5-dithiobis-2-nitrobenzoic acid using a commercial kit (Diacron, Italy). Plasma retinol, tocopherol, and β-carotene were extracted with hexane and analysed by reversed-phase HPLC using Spherisorb ODS-2, 3 µm, in a 150 × 4.6 mm column (Alltech, Deerfield, IL, USA); a UV detector set at 325 nm (for retinol), 290 nm (for tocopherol), or 460 nm (for β-carotene); and 80:20 methanol: tetrahydrofuran as the mobile phase.

K-EDTA tubes were processed using a clinical autoanalyzer. Samples were analysed within 2 h of collection using an ADVIA 2120 Haematology System machine (Siemens, Germany). The blood parameters analysed were the following: red blood cell count (RBC), hematocrit value (HCT), haemoglobin concentration (HGB), platelet count (PLT), total white blood cell (WBC) count, WBC differential count for neutrophils, eosinophils, basophils, lymphocytes, and monocytes as an absolute number.

4.3.4 Statistical Analysis

Significance analysis of the environmental parameters, milk yield, milk composition, biomarkers of oxidative stress, blood cells, and plasma metabolites at different time points was conducted using one-way ANOVA supplemented by Dunnett's multiple comparisons post-hoc test compared to T4, with analysis performed using GraphPad Prism v8.4.2 (GraphPad Software, Inc., La Jolla, CA, USA). Results are presented as the mean ± standard deviation (SD). A statistically significant difference was defined as $p < 0.05$ and highly significant at $p < 0.01$.

4.4. Results

4.4.1. Environmental Conditions

Mean values of daily ambient temperature, relative humidity, and THI measured during the weeks of time 1, time 2, time 3, and time 4 in the period of April–July 2022 are presented in Table 3. The average daily temperature (30.45 ± 1.29 °C) and THI (80.15 ± 0.70) were significantly higher ($p < 0.001$) in T_4 than T_1, T_2, and T_3 (Figure 1).

Table 3. Mean weekly Temperature-Humidity Index (wTHI) values and number of days with THI mean above 75 points during the week previous the blood and milk sampling day (T1, T2, T3, and T4) in Modicana mild-lactating cows (Ragusa, Italy, spring–summer 2022).

Week	**Time**	**T °C min**	**T °C max**	**T °C mean**	**Hr (%) min**	**Hr (%) max**	**Hr (%) mean**	**wTHI min**	**wTHI max**	**wTHI mean**	**n. Days THImax 75–78**	**n. Days THImax > 78**
20–27 May	T_2	19	25	22	43	82	64	63	74	68	1	2
10–17 June	T_3	20	27	23	50	88	72	65	79	71	3	4
19–26 July	T_4	27	35	30	28	83	59	71	91	80	0	7

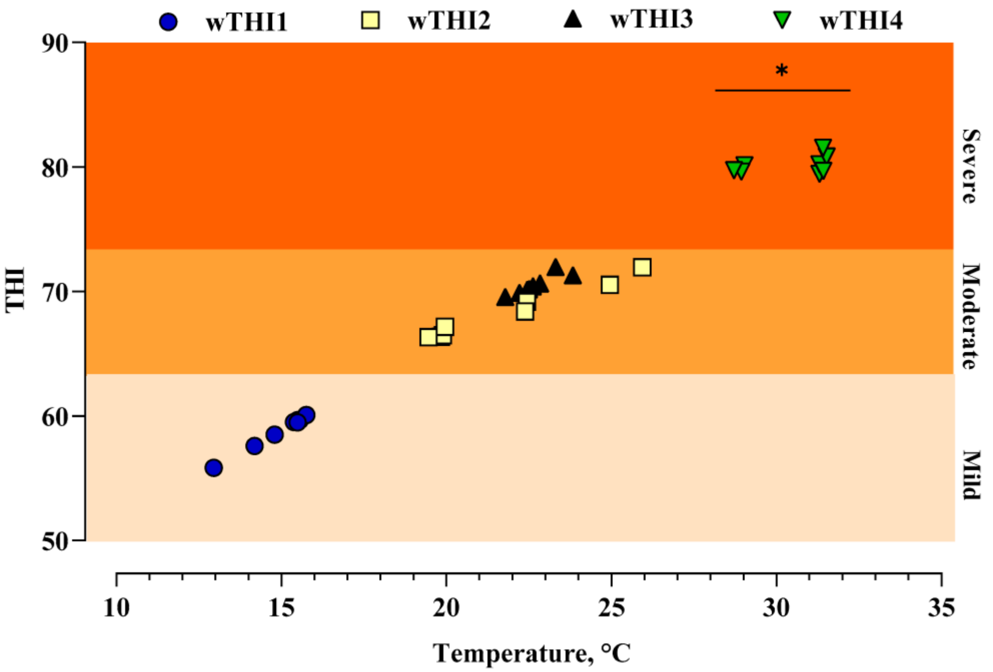

Figure 1. Mean daily values of weekly temperature (°C) and weekly temperature-humidity in- dex (wTHI) during the experimental period are presented. Asterisk indicate statistical difference compared to previous periods (* $p < 0.001$).

4.4.2. Milk Parameters

As shown in Table 4, although no differences were detected between groups for milk yield, protein, and lactose concentrations, the milk fat concentration was significantly decreased in the THI_3 group ($p < 0.05$), whereas the highest value was observed at THI_1, when Modicana cows were in a condition of thermal comfort (THI = 55).

Table 4. Effect of temperature-humidity index (THI) increase on milk performance of Modicana mild-lactating cows.

	wTHI Periods				
Item	**$wTHI_1$ 55**	**$wTHI_2$ 68**	**$wTHI_3$ 71**	**$wTHI_4$ 80**	***p*-Values**
Milk yield, kg/d	13.45 ± 3.92	11.97 ± 3.95	13.49 ± 3.42	11.33 ± 2.77	0.38
Fat, g/100 g	4.72 ± 0.81	3.64 ± 1.41	3.35 ± 1.40	3.73 ± 0.62	<0.05
Proteins, g/100 g	3.94 ± 0.33	3.84 ± 1.18	3.48 ± 1.30	3.73 ± 0.62	0.67
Caseine, g/100 g	3.01 ± 0.28	2.85 ± 0.32	2.83 ± 0.30	2.96 ± 0.19	0.32
Lactose, g/100 g	4.64 ± 0.22	4.79 ± 0.16	4.89 ± 0.34	4.82 ± 0.17	0.07

4.4.3. Haematology, Biomarkers of Energy, Muscle Body Mass, and Liver Function

As shown in Table 5, there was a significant decrease in both RBC and WBC counts ($p < 0.05$). The RBC count was significantly lower during wTHI4 compared to wTHI1 and wTHI2 ($p < 0.05$ and $p < 0.01$, respectively). WBC count, particularly eosinophils, was significantly higher during wTHI2 compared to wTHI4 ($p < 0.05$). The number of monocytes significantly increased ($p < 0.01$), with significant differences observed at wTHI4 compared to wTHI1 ($p < 0.05$) and wTHI2 ($p < 0.01$). Plasma glucose and NEFA decreased significantly ($p < 0.001$ and $p < 0.01$, respectively), while BHB and creatinine significantly increased ($p < 0.05$ and $p < 0.001$, respectively) (see Table 6). The highest glucose values were observed in wTHI1 and the lowest in wTHI4 ($p < 0.01$), while the lowest BHB values were in wTHI1 ($p < 0.05$) and the highest creatinine values were in wTHI4 ($p < 0.01$).

Table 5. Effects of the weekly value of the temperature-humidity index (wTHI mean) on haematology of Modicana mild-lactating cows.

	wTHI Periods				
Items	**$wTHI_1$ 55**	**$wTHI_2$ 68**	**$wTHI_3$ 71**	**$wTHI_4$ 80**	***p*-Values**
PCV, %	28.79 ± 3.86	32.02 ± 4.30	29.93 ± 7.39	28.93 ± 4.18	1.01

RBC, M/µL	6.57 ± 0.82 *	7.19 ± 0.89 **	6.21 ± 1.97	5.30 ± 0.69	<0.01
Hb, g/dL	9.33 ± 1.16	10.02 ± 1.20	9.59 ± 1.36	8.93 ± 1.14	0.18
PLT, K/µL	396 ± 91	299 ± 66	366 ± 99	360 ± 115	0.09
WBC, K/µL	10.57 ± 2.49	12.83 ± 1.57 *	10.81 ± 1.82	10.45 ± 1.66	<0.05
Neutrophils, K/µL	4.21 ±1.42	5.60 ±1.23	4.56 ±1.66	4.54 ± 1.33	0.10
Lymphocytes, K/µL	6.06 ± 1.15	5.12 ±1.58	5.63 ±1.18	4.08 ± 1.18	0.14
Eosinophils, K/µL	0.98 ± 0.51	1.66 ±1.12 *	0.98 ± 0.80	0.76 ± 0.37	<0.05
Monocytes, K/µL	0.23 ± 0.06 *	0.27 ± 0.05	0.20 ± 0.10 **	0.34 ± 0.12	<0.01

* Dunnette multiple comparisons test differences vs. $wTHI_4$ (* $p < 0.05$; ** $p < 0.01$).

Table 6. Effects of the weekly value of temperature-humidity index (wTHI mean) on energy, body mass, and liver function biomarkers in the plasma of Modicana mild lactating cows.

	wTHI Periods				
Items	**wTHI$_1$ 55**	**wTHI$_2$ 68**	**wTHI$_3$ 71**	**wTHI$_4$ 80**	***p*-Values**
Glucose, mmol/L	3.94 ± 0.23**	3.57 ± 0.28	3.72 ± 0.28 *	3.41 ± 0.33	<0.001
Fructosamine, µMol/L	261 ± 24	277 ± 27	277 ± 20	281 ± 17	0.17
NEFA, mmol/L	0.06 ± 0.02	0.05 ± 0.01	0.09 ± 0.05	0.05 ± 0.01	<0.01
BHB, mmol/L	0.45 ± 0.14 *	0.51 ± 0.10	0.61 ± 0.20	0.62 ± 0.17	<0.05
Creatinine, mmol/L	87.19 ± 5.37 **	102 ± 7.32	105 ± 13.2	109 ± 7.83	<0.001
Urea, mmol/L	4.88 ± 0.93	4.89 ± 0.75	5.26 ± 1.08*	4.83 ± 0.81	0.10
Cholesterol, mmol/L	4.05 ± 0.90	4.33 ± 0.37	3.40 ± 0.92	3.75 ± 1.03	0.10
Total protein, g/L	85.8 ± 6.8	90.5 ± 7.7	92.5 ± 0.37	91.06 ± 5.52	0.08
Albumin, g/L	31.41 ± 3.14	33.26 ± 3.59	31.37 ± 2.33	31.13 ± 3.38	0.33
A/G ratio	0.59 ± 0.09	0.59 ± 0.11	0.52 ± 0.07	0.52 ± 0.07	0.10
Bilirubin, µmol/L	1.00 ± 0.35	1.10 ± 0.23	1.19 ± 0.78	1.20 ± 0.79	0.29

* Dunnette multiple comparisons test differences vs. wTHI$_4$ (* $p < 0.05$; ** $p < 0.01$).

4.4.4. Oxidative Biomarkers and Antioxidant Parameters in Plasma

As shown in Figure 2, significant changes were observed in the levels of ROM, which significantly increased ($p < 0.05$), and in the activity of PON and AOPP levels, which significantly decreased ($p < 0.05$) as an effect of incremental wTHI. A significant and progressive decrease of carotenoids was found during the experimental period ($p < 0.001$; Figure 3). Dunnett’s multiple comparison post-hoc test revealed significant differences for each group compared to wTHI$_4$.

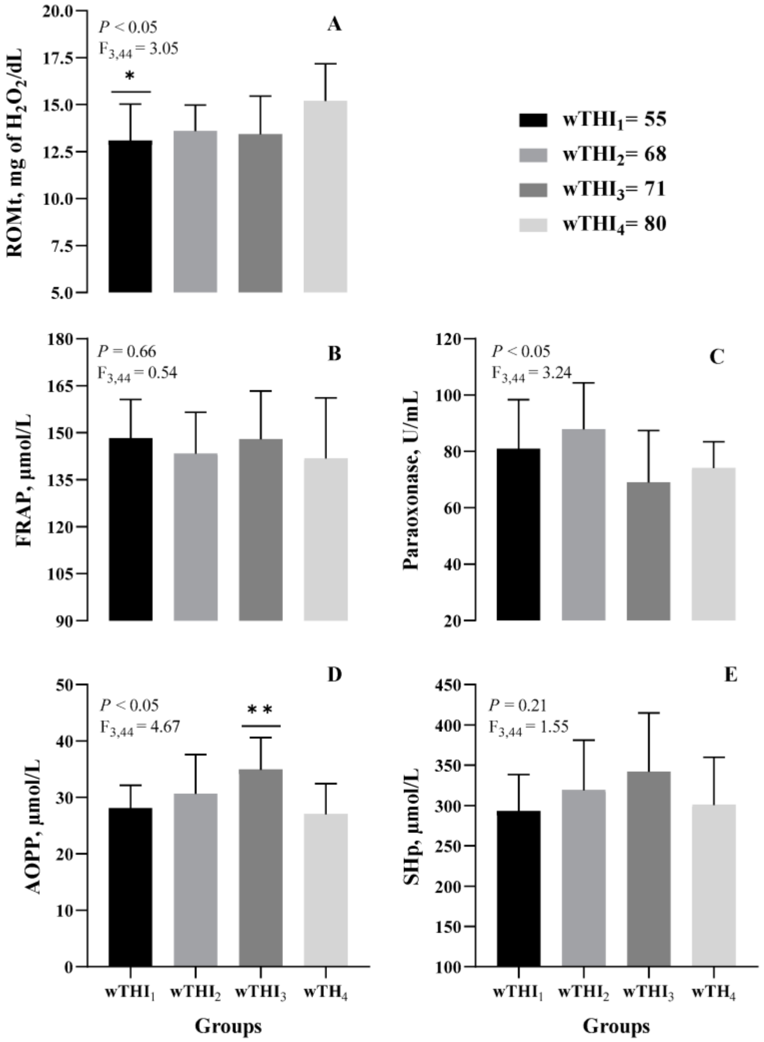

Figure 2. Effect of weekly temperature-humidity index (wTHI) on plasma biomarkers related to oxidative stress and antioxidant defence in Modicana mild lactating cows (**A**) reactive oxygen metabolite totals (ROMt), (**B**) ferric reducing ability of plasma (FRAP), (**C**) paraoxonase (PON), (**D**) advanced oxidation protein products (AOPP), (**E**) plasma thiol groups (SHp). Asterisks indicate statistical differences vs. THI$_4$ (* $p < 0.05$; ** $p < 0.01$).

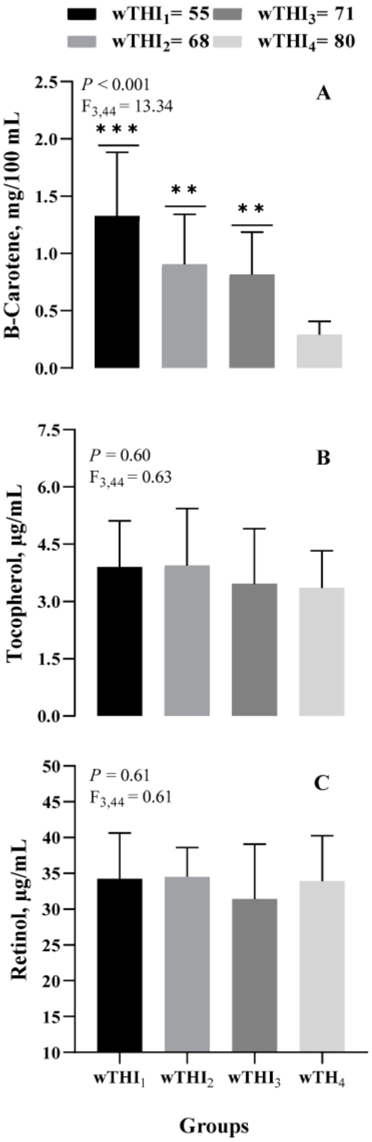

Figure 3. Effect of weekly temperature-humidity index (wTHI) on lipid-soluble antioxidant pro-vitamin ((**A**) β-carotene) and vitamins ((**B**) tocopherol, (**C**) retinol) in the plasma of Modicana mild lactating cows. Asterisks indicate statistical differences vs. wTHI4 (** $p < 0.01$; *** $p < 0.005$).

4.5. Discussion

This study aimed to evaluate the effects of the incremental temperature-humidity index (THI) on haematological values, biochemical metabolites, and the balance between oxidative markers and the antioxidant defence system in the plasma of Modicana dairy cows. A common belief among breeders of the Modicana breed is that cows are not affected by heat stress because it is a rustic breed. Confirming these beliefs, Modicana cows showed no differences in milk yield from April to July. This is an interesting result, since several previous studies demonstrated how heat stress decreases the production of milk (West, 2003; Bohmanova et al., 2007). In fact, it is well known that high yielding cows are more challenged by heat stress than lower yielding cows (Spiers et al., 2004), since the genetic selection for high milk production reduces their thermoregulatory ability (El-Tarabany et al., 2018). On the other hand, Modicana cows, being less productive, can better cope with heat stress (producing less metabolic heat). Numerous studies had previously demonstrated the changes in milk composition in dairy cows under heat stress (Bernabucci et al., 2014; Hill and Wall, 2015; Liu et al., 2017; El-Tarabany et al., 2018)

In the current study, milk protein and lactose concentration were not affected by different levels of THI, and this was in accordance with previous studies in buffalo and cow milk (Nasr, 2016; Li et al., 2021). However, the milk fat content decreased when THI increased, as consistent with previous studies (Lambertz et al., 2014; Zhang et al., 2014; Li et al., 2021).

This could be related to a decrease in dry matter intake in cows during the hottest period, which resulted in a decreased intake of fibre (Bouraoui et al., 2002) probably as a consequence of reduced acetate production in the rumen. Unfortunately, we have no data on dry matter intake.

The RBC count decreases in T_4, and this change could be an effect of the destruction of erythrocytes by oxidative stress (Pasciu et al., 2023). If a slight upward trend in bilirubin levels were to have been found, this explanation could be plausible. The decrease of RBC but not of PCV could indicate that a slight dehydration, masked by the concomitant presence of anemia, occurred in these animals. The concomitant but not significant increase in total proteins, without alteration of the A/G ratio, also confirms this hypothesis. Although A/G is within the range, it shows lower values than those previously reported (Alberghina et al., 2011). The fact that there is fibrinogen in the plasma compared to the serum could explain this result. Heat stress has been proven to decrease the activity and quantity of specific immune cells, such as neutrophils and lymphocytes, making cows more susceptible to infections and illness (Giannone et al., 2023). Eosinophils respond differently to heat stress, and it has been documented that there is a reduction in the eosinophil counts in sheep exposed to high temperatures (Gomes Da Silva et al., 1992). A significant increase in monocytes has been found. Monocyte numbers are variable in cattle and are thus not a sensible indicator for a specific disease, but usually monocytosis has been observed during acute stress

(Alhidary et al., 2012; Roland et al., 2014). Monocytes play a pivotal role in defence against infection or injury, and they are responsive to heat stress in humans (Oehler et al., 2001).

The increased plasma creatinine concentrations observed in this and earlier studies (Lambertz et al., 2014; Lamp et al., 2015) might be primarily due to a reduction in renal clearance rather than increased muscle catabolism, as urea levels remained unchanged. However, heat stress slightly increased levels of fructosamine, but in our study, it failed to be a marker of stress. Recently, elevated fructosamine levels have been reported in dairy cows under chronic stress (Grelet et al., 2022).

Increased BHBA blood levels, as well as decreased glucose levels, suggest a negative energy balance (Akbar et al., 2015). Energy balance is the difference between energy consumed and energy used for both maintenance and production. When energy expenditure exceeds intake, this results in a state of Negative Energy Balance (NEB) (Giurgiu et al., 2024). For heat stress metabolism during mid-lactation, there is no increase in NEFA concentrations; this finding agrees with some previous studies (Rhoads et al., 2009a; Wheelock et al., 2010a).

Oxidative metabolism of fatty acids is essential for creating energy for various biological functions, but it also ROM, or free radicals, that can be destructive to cells and tissues (Schönfeld and Wojtczak, 2008). When ROMs are produced faster than they can be neutralized by antioxidant mechanisms, oxidative stress can result. There are increasing numbers of reports that suggest the involvement of ROM in a variety of pathologic and physiological events. A tendency for an increase in ROM levels has been found in sheep as an effect of thermal stress (Chauhan et al., 2014). In transition cows, those with a high body condition score showed neither a change in plasma ROM levels (Bucktrout et al., 2021) nor higher levels (Bernabucci et al., 2005). In Holstein cows exposed to a nutritional and environmental stress challenge, the variation in ROM was significant (Cavallini et al., 2021). To the best of the author's knowledge, an increase in ROM levels related to heat stress in dairy cows, as observed in this study, has not yet been reported. Bernabucci et al. (2002) found that the effect of heat stress (THI = 75) on the oxidative status in transition dairy cows by using plasma markers does not give enough information to reach definitive conclusions. The difference found in this study compared to the previous could be related to the different breed, stage of lactation, or, more likely, to the higher values of THI reached.

PON is a calcium-dependent esterase that is suggested to play a protective role under oxidative stress. However, there is no information in the literature about the alteration of PON levels in thermal oxidative stress. Recent studies have reported that PON acts as an antioxidant enzyme, decreasing oxidative stress by preventing the formation of free radicals. There is an inverse relationship between lipid peroxidation and PON activity (Aviram, 1999). This could explain the significant reduction in PON activity found at higher THI value. In cows, a reduction in the ability of the liver to cope with increased metabolic demand near parturition can be diagnosed by a decrease in baseline plasma PON levels (Bionaz et al., 2007).

In dairy heifers, PON activity was significantly decreased after calving in both summer and winter groups (Benzie and Strain, 1996). In rats, a decrease was found after exercise with increasing temperature (Şıktar et al., 2011), but, to our best knowledge, this is the first report of a significant decrease in PON during thermal stress in mild lactating cows.

Higher concentrations of AOPP in T_3 suggest that these animals suffered oxidative stress and damage to proteins when exposed to heat stress. The observed elevation of AOPP is of particular interest, as AOPP is a marker of protein oxidation and is also considered to mediate proinflammatory responses (Celi et al., 2012). In dairy cows, AOPP concentrations are associated with embryonic losses and are considered an indicator of acute inflammation and oxidative stress (Celi et al., 2012). It has been observed that the concentration of AOPP increases in dairy cows when they are fed maize silage (Celi et al., 2012) and in growing dairy calves (Celi and Robinson, 2010), possibly due to lower levels of antioxidants in silage. An increase in AOPP as a response to thermal stress in sheep has been found (Chauhan et al., 2014). FRAP and SHp seem not to be clearly reflective of their involvement in the response to thermal stress in our study conditions.

However, the significant decrease in carotenoids due to oxidative stress underscores the necessity of providing carotenoid supplementation during thermal stress. Vitamin E and β-carotene blood levels varied considerably between lactation stages and between countries (Celi et al., 2011). In Holstein cows, levels of tocopherol and carotenoids are higher during mid-lactation than during other lactation stages (Mary et al., 2021). The β-carotene levels found in this study are very low in T_4. This is likely because carotenoid intake declined as fresh forage decreased from T1 to T4, and also because β-carotene is more heavily utilized as an antioxidant in response to the increase in NEB and ROM. A higher blood β-carotene level at the time of artificial insemination increased the pregnancy rate and reduced pregnancy loss (Madureira et al., 2020). Since a large proportion of the cows in the present study were considered deficient in β-carotene, dietary supplementation of this nutrient appears to deserve more attention in practical nutrition. Carotenoid supplementation may also help modulate the antioxidant effects of PON (Otocka-Kmiecik, 2022).

4.6. Conclusions

In summary, our results clearly show that the progressive increase in THI causes an imbalance between oxidative markers and the antioxidant defence system in Modicana cows, leading to a decrease in milk fat content. The common opinion that Modicana cows are not affected by heat stress must be reconsidered in light of these results. Limitations should be acknowledged when interpreting the substantive findings of this study: body temperature measurements of the studied cows consistently remained within normal ranges, and observations of heat stress behaviours were not conducted. Nevertheless, the results obtained indicate the necessity for implementing environmental management and nutritional supplementation in dairy cow housing in Sicily to assist cows in adapting to increasingly frequent thermal stress.

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CHAPTER 5

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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INTERPRETIVE SUMMARY

Dry Grape Extract In Heat Stressed Dairy Cows. The adoption of feeding strategies can help cows meet their nutritional needs during heat stress reducing its negative impact and supporting their recovery through this critical period. This study investigated the impact of supplementing a rumen-protected grape extract, rich in polyphenols, on production and immunometabolism of heat-stressed dairy cows. Cows supplemented with a rumen-protected grape extract showed an improved body temperature and panting, milk production, phagocytic activity, liver functionality, and inflammatory response, compared to non- supplemented cows. These findings highlight rumen-protected grape extract supplementation as an effective strategy to mitigate the adverse effects of heat stress, enhancing cow welfare and maintaining productivity during summer.

5.1. 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) on milk performance, rectal temperature (RT), behavior, immunometabolism, rumen fermentation, and innate immune response under naturally ocurring HS condition (THI > 72). Thirty cows were balanced by days in milk (DIM), milk yield (MY), and parity, and randomly assigned to receive either 470 mg/cow/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 goups were cooled daily using forced ventilation and fans. Temperature and humidity index (THI), 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 rectal temperature was measured. The NG-BPO cows exhibited greater milk production, fat corrected milk, energy corrected milk, and yields of fat and protein, alongside a lower somatic cell count compared to CTR cows. NG-BPO cows exibited improved thermoregulatory response, reflected by significant 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 to CTR cows. These findings were supported by higher levels of pro-inflammatory cytokine TNF-α and IL-1 β during peak HS periods, compared with CTR cows. In addition, the NG-BPO group had lower circulating platelets and eosinophils counts compared to 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, it 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 heat stress, by enhancing cow performance and welfare during periods of elevated environmental temperatures.

Key words**:** heat stress; grape polyphenols; antioxidant; inflammation.

5.2. Introduction

Heat stress (**HS**) is one of the major challenges to the global livestock industry, especially for dairy cattle. In fact, compared to other livestock species, dairy cattle are more susceptible to HS, due to genetic selection for high milk yield in which leads to increased metabolic heat production (Baumgard and Rhoads, 2012; Loor et al., 2023). In dairy cattle, the thermo-neutral zone —the range in which they do not need to expend extra 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. When Temperature-Humidity Index (**THI**) is 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 per day 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 the 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's 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 a decrease in dry matter intake (DMI), which account for only 35-50% of the heat stress-induced decrease in milk synthesis. (Rhoads et al., 2009b; 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 in the Italian context the economic losses due to HS in Italian dairy farms are estimated at $23.57–$43.98 per farmer per day, mainly from reduced milk quality rather than yield (Moore et al., 2023). However, economic losses reflected not only the 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 (ROS) exceed the capacity of antioxidant defense mechanisms, impairing immune function and increasing the susceptibility of cows to disease (Alberghina et al., 2024).

The impairement of the animal’s immune status is caused, in part, by a reduction in neutrophil phagocytosis and lymphocyte proliferation (Tejaswi et al., 2020), which play an important role 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 cytokine and immunoglobulin (Safa et al., 2019).

The most adopted method to minimize the negative impact of HS is the use of evaporative cooling, fans and water sprinklers (Mader et al., 2007). However, these heat abatement strategies have 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, 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 improves productivity of dairy cows under HS (Sun et al., 2019). The inclusion of polyphenols in dairy cow diets may offer a valuable stategy to reduce oxidative stress associated with HS, due to its 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) 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 heat stress and during summer heat waves.

5.3. Materials and methods

5.3.1. Animals 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 Fattoria Demetra Srl Soc. Agricola commercial farm in Calabria (South Italy) during the peak summer (July-August), under natural heat stress 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 by milk yield, parity, and DIM: 1) standard milking cow diet (**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; Nor-Feed, France; **NG-BPO**, n = 15).

The supplement consisted in 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 supplementation of NG-BPO was mixed with corn meal (100 g) and top-dressed individually onto total mixed ration (TMR) once a day in the morning at 0700 h and provided a daily dietary supply of 282 mg 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 one empty space between each animal, as the pen where they were housed had a feed bunk with 65 headlocks. Each cow remained individually head-locked until the top-dress was completely consumed and was monitored throughout the entire process. Approximately one 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, since each cow took approximately 15 seconds 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 the Table 1.

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

Ingredients	**% of DM**
Grass hay	25.4
Alfalfa hay, second or later cuts	18.1
Corn grain, ground, dry	21.6
Commercial lactating concentrate mix[1]	20.0
Steam-flaked corn	9.4
Molasses	2.2
Minerals and vitamins 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
NE_L [4], Mcal/kg of DM	1.65

[1] Contained: solvent-extracted flour of toasted soybean, solvent-extracted flour of sunflower, dried stillage corn, whole cottonseeds, 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 D3, 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 rume-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).

Although all cows were housed in a single pen, supplementation was managed at the individual level in order to allow the accurate monitoring of the two treatments. Cows were milked 2 times daily at 05:00 and 17:00 h in an 8-herringbone milking parlor. All the animals of both groups were cooled all days by forced ventilation in the free-walking resting area (straw-bedded) automatically turned on when the 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 the THI exceeded 64, whereas soakers automatically turned on when THI exceeded 68 and were activated for 1 min at 5 min intervals from 08:00 to 20:00 h of 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, Calvisano, Italy). Ambient temperature and relative humidity to calculate the THI were recorded every 15 min by 3 probes installed in the feeding and resting area and in the milking parlor (CMP Animal Welfare, Calvisano, Italy). 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.

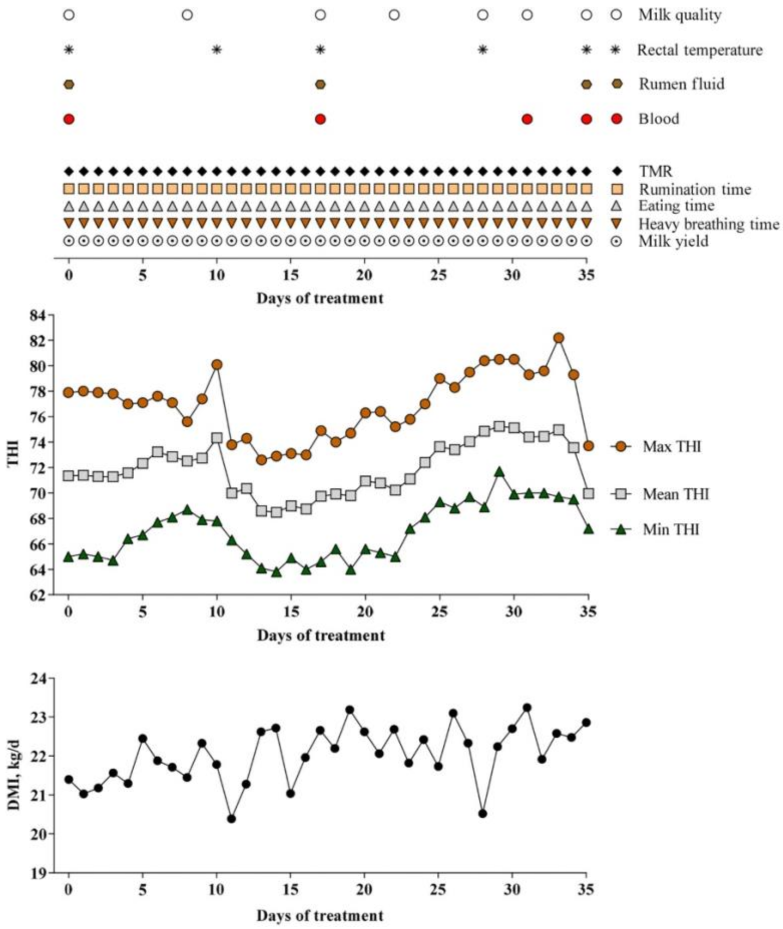

Figure 1. Experimental design with sampling time points, THI trend, and the daily DMI average of all lactating cows enrolled in the trial under the same environmental condition, throughout the entire period. Mid-lactating Fleckvieh cows during summer were divided into 2 groups and fed for 35 d either a standard diet (CTR) or diet supplemented with rumen-protected dry grape extract at 470 mg/head/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.

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

5.3.2. Animal Behavior Recordings and Rectal Temperature Measurement

All cows were equipped with the SenseHub® sensors collar (MSD Animal Health) that monitored and transmitted data to the installed SenseHub® dairy system antenna during the entire period of the trial. The SenseHub® sensors collar 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 dataset included the parameters 'Rumination', 'Eating', and 'Over Heat' (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 min/h. Finally, processed sensor collar data were organized as min/h for all the parameters considered and then a time daily 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 (08:00 h), at 14:00 h, and immediately after the evening milking (20:00 h). The RT was recorded using a clinical digital thermometer (TFA Dostmann Thermometer VET 112, Germany) inserted to a depth of approximately 10 cm, and measurements were always performed by the same operator to minimize variability.

5.3.3. Milk Yield, Sample Collection, and Analysis

Milk yield was automatically recorded at each milking (twice daily, and 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 two 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 total mixed ration (TMR) meals provided to cows throughout the day, as both milk samples were taken from cows that were fed TMR over a single day rather than over two days. Milk samples were analyzed separately (evening and morning) for fat, protein, lactose, casein, urea, total milk solids, non-fat milk solids, citric acid, and free fatty acids by mid-infrared spectroscopy using the Milkoscan FT2 (Foss Electric A/S, 3400 Hillerød, Denmark) and for somatic cell count using the Fossomatic^{TM} FC (Foss Electric, Hillerød) at the DALILAB laboratory of the Department of Veterinary Sciences in 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.

5.3.4. Blood Sampling for Plasma and Hematology Analysis

Blood samples were collected by jugular venipuncture into 10-mL heparinized and K_3EDTA 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_3EDTA 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 one hour of collection, and subsequently processed as described by Lopreiato et al. (2021) for the assessment of a wide blood-based biomarkers profile in the 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 (kit Ranbut, Randox Laboratories Ltd., Crumlin, UK). Electrolytes ($Na+$, $K+$, and $Cl-$) were detected by the potentiometer method (ion selective electrode connected to ILAB 650). 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 Ferrè 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); the 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 the plasma sample 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), and advanced oxidation protein products (AOPP) as described by Hanasand et al. (2012). Thiol group levels (SHp) were measured using the 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 two fractions. Plasma from blood samples at 17 and 31 d of treatment was also assessed for interleukin (IL) 1β, IL-6, IL-10, interferon gamma (IFN-γ), and tumor necrosis factor (TNF) α. These biomarkers were measured by commercial ELISA kits method 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 (HRP) 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 the plate reader Victor-X3™ (Perkin Elmer®). The concentration of each cytokine was calculated by comparing the OD of the samples to the standard curve.

Blood samples collected into K_3EDTA were stored in a thermos-insulated container at room temperature and analyzed within 3 h for a complete blood count by an automatic clinical hematology device (Sysmex XN-1000, Japanese Sysmex company, 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, mean platelet volume, platelet crit, lymphocytes, monocytes, neutrophils, eosinophils, and basophils.

5.3.5. Phagocytosis capacity of neutrophil and monocyte

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, two whole blood aliquots of 200 µL were transferred into 2 tubes: one for the control and one with the addition of 80 µL of fluorescently labeled *E. coli*. The tubes were incubated at 38.5°C for 10 min and then cooled in an iced bath for 10 minutes. The erythrocytes were hypotonically lysed adding 800 μL of iced Milli-Q® PF water and then quickly added 200 μL of 5X Phosphate-Buffered Saline (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 1X and centrifuged at 990 × g for 5 min at 4°C. Cells were resuspended with 200 μL of PBS 1X and analyzed with flow cytometry (Attune NxT Flow Cytometer,). Data were analyzed according to Scatà et al. (2024) using flow-cytometer analysis software (BD Accuri™ C6 Plus Software) to obtain the percentages of phagocytic cells.

5.3.6. Rumen Samples and Gas Chromatography for VFA Analysis

At 0, 17, and 35 d of treatment, rumen fluid was collected 4 hours 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 everytime before use with standard buffer solutions (pH 4.0, 7.0 and 10.0) to ensure accuracy (XS Basic, XS Instruments, Italy). 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 minutes to obtain a clear supernatant for analysis. The concentrations of VFAs 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 auto-analyzer (ILAB-650), after that, NH_3-N was obtained dividing the urea value by 2 (approximation). D- and L-lactic acids were measured using a D-/L-lactate assay kit (Megazyme International Ltd.), adhering to the manufacturer's instructions.

5.3.7. 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), and rectal temperature 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, whereas individual cows were included as random effect. Phagocytic activity of neutrophils and monocytes were analyzed with the GLM procedure of SAS where the model included the only fixed effect of treatment (CTR and NG-BPO), since blood samples for this assessment were collected only at 35 d of treatment. For repeated measurements, four 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 GLIMMIX procedure, they were back-transformed. All means were compared using the PDIFF statement of SAS and Tukey-Kramer's adjustment post-hoc was applied. Statistical significance was declared at $P \leq 0.05$ and tendencies at $0.10 \geq P > 0.05$.

Finally, two-phase segmented regressions were performed on the least square means of milk yield and heavy breathing 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 $P \leq 0.05$.

5.4. Results

5.4.1. Milk production and quality

Results of milk performance are reported in Table 2.

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

	Treatment[1]			***P*-value**[3]		
Item	**CTR**	**NG-BPO**	**SEM**[2]	**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
Non-fat 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 cells count, x 10^3 cells/mL	112.36	87.85	27.03	0.05	0.15	0.49

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[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] Greatest standard error of the mean.

[3] 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.

Dietary supplementation of Nor-Grape BP-O affected milk yield (MY), which resulted overall greater in NG-BPO group compared with CTR group (29.3 vs. 27.4 ± 0.25 kg/d for NG-BPO and CTR, respectively; Trt; $P < 0.01$). In Figure 2A is reported 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 identification of threshold 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 every increase of THI unit above the threshold. Regarding NG-BPO cows, the THI breakpoint identified was 72.83, above which cows decreased the MY at a rate of -1.42 kg/d for every unit of THI above the threshold. The Fisher-type Z-transformation test revealed that the correlation between THI and MY in the two-phase segmented regression was similar between CTR group (adj. $r = 0.51$; adj. $R^2 = 0.26$) and NG-BPO group (adj. $r = 0.49$; adj. $R^2 = 0.24$), with a *P*-value of 0.46 (Z value = 0.11).

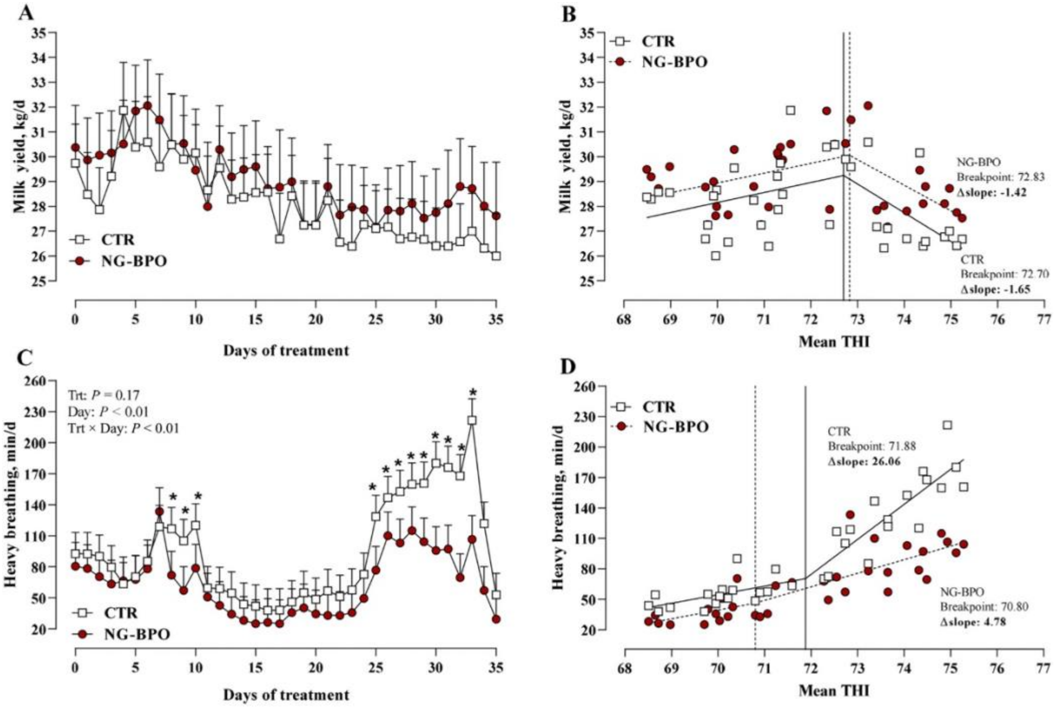

Figure 2. Trend milk yield (A) with segmented regressions relative to daily mean THI (B) and trend of heavy breathing (C) with segmented regressions relative to daily mean THI (C) of mid-lactating Fleckvieh cows fed either a standard diet (CTR, n = 15) or diet supplemented with rumen-protected dry grape extract at 470 mg/head/day (NG-BPO, n = 15) for 35 d during a natural heat stress 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 (A and C). Vertical lines indicate breakpoint at which milk yeld (B) and heavy breathing (D) changed significantly and abruptly in NG-BPO cows (---) and in CTR cows (—). 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).

Among results of milk quality (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 CTR group. Additionally, NG-BPO cows showed overall lower somatic cell count (NG-BPO: 87.85 *vs*. CTR: 112.36 ± 27.03 x 10^3 cells/mL; Trt; $P = 0.05$), compared with CTR group.

5.4.2. Rectal Temperature and Behavior Response

In Table 3 are reported results of rectal temperature, heavy breathing, rumination, and eating time.

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

	Treatment[1]			***P*-value[3]**		
Parameter	**CTR**	**NG-BPO**	**SEM[2]**	**Trt**	**Time**	**Trt * Time**
Rectal Temperature, °C						
08:00 h	38.75	38.60	0.12	0.07	0.01	0.05
14:00 h	38.73	38.79	0.17	0.54	< 0.01	0.87
20:00 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/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] Greatest standard error of the mean.

[3] 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

Among the measurement of heavy breathing (HB), a significant interaction effect (Trt * Time; $P < 0.01$) was observed, where NG-BPO cows showed a lower HB compared with 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 the HB was 71.88, whereby HB began rising at a rate of 26.06 min/d for every unit increase THI above the threshold. A different trend was observed for NG-BPO cows, where the THI breakpoint was lower (70.80) compared twith 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 two-phase segmented regression was was significantly stronger in the CTR group (adj. r = 0.94; adj. $R^2 = 0.88$) compared with the NG-BPO group (adj. r = 0.84; adj. $R^2 = 0.70$), with a *P*-value of 0.02 (Z value = 2.07).

Overall, the parameter of rectal temperature (RT) showed a tendency at 0800 h in the NG-BPO cows compared to 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 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) d of treatment ($P < 0.05$) in the NG-BPO cows compared with CTR cows (Figure 3).

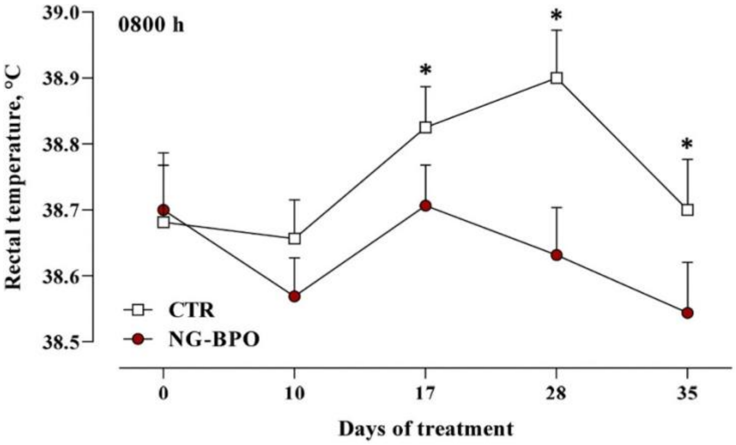

Figure 3. Rectal Temperature (°C) of mid-lactating Fleckvieh cows fed either a standard diet (CTR) or diet supplemented with rumen-protected dry grape extract at 470 mg/head/day (NG-BPO) for 35 d during a natural heat stress exposure in summer. Differences between groups within each time point ($P \leq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

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

5.4.3. 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.

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 diet supplemented with rumen-protected dry grape (NG-BPO) for 35 d during a natural heat stress exposure in summer.

	Treatment[2]			***P*-value**[4]		
Biomarker[1]	**CTR**	**NG-BPO**	**SEM**[3]	**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] BOHB = beta-hydroxybutyrate; NEFA = free fatty acids; PON = paraoxonase; GOT = 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.

[2] Cows 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.

[3] Greatest standard error of the mean.

[4] 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.

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 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 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 NG-BPO group compared with 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 resulting 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.

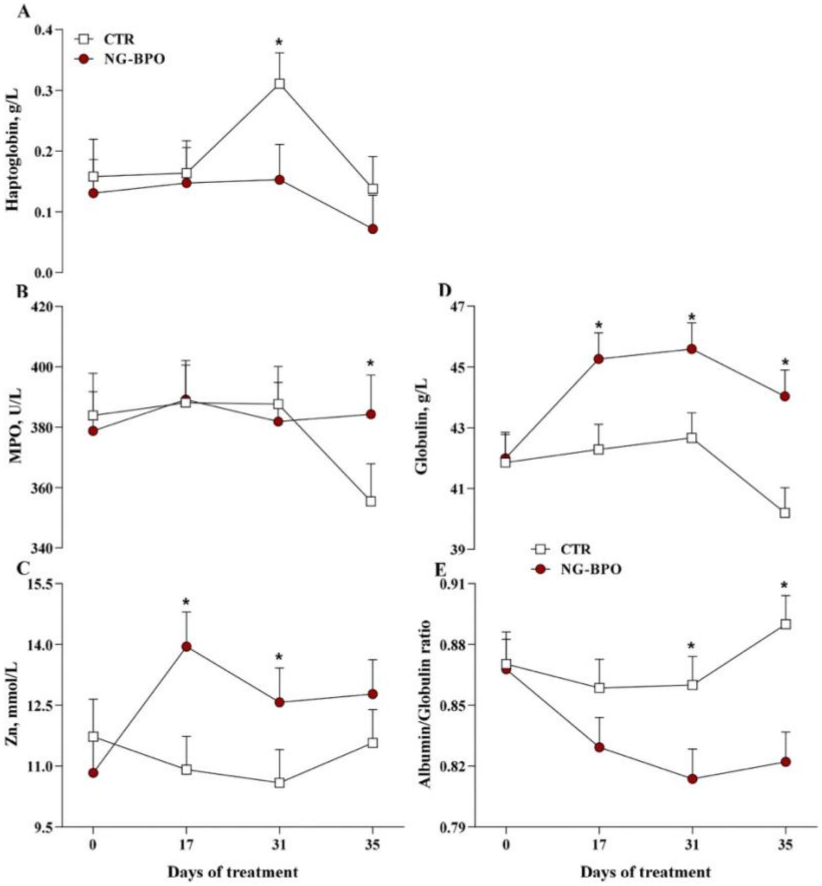

Figure 4. Plasma haptoglobin (A), myeloperoxidase (MPO, B), zinc (Zn, C), globulin (D), and albumin-to-globulin ratio (E) of mid-lactating Fleckvieh cows fed either a standard diet (CTR) or diet supplemented with rumen-protected dry grape extract at 470 mg/head/day (NG-BPO) for 35 d during a natural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

5.4.4. Plasma Cytokines

Table 5 summarizes the differences between NG-BPO and CTR group in blood cytokines.

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

	Treatment[1]			***P*-value**[3]		
Biomarker	**CTR**	**NG-BPO**	**SEM**[2]	**Trt**	**Time**	**Trt * Time**
IL-1β, pg/mL	22.70	26.21	2.83	0.04	0.28	0.57
IL-6, pg/mL	6.58	6.77	0.62	0.68	0.01	0.68
IL-10, pg/mL	61.37	39.17	34.06	0.38	0.44	0.63

IFN-γ, pg/mL	22.70	21.97	1.70	0.57	0.60	0.90
TNF- α, pg/mL	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/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] Greatest standard error of the mean.

[3] 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 .

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 resulted, 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, and IFN-γ ($P > 0.05$; Table 5).

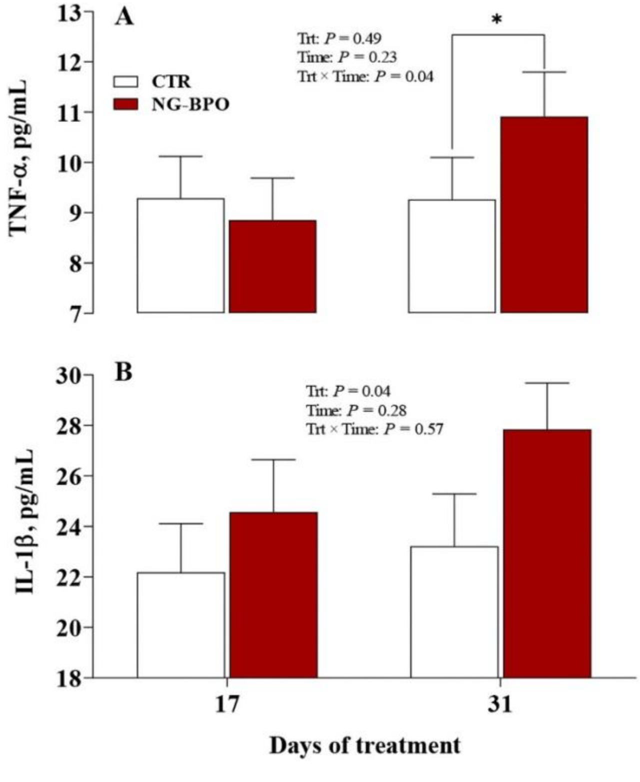

Figure 5. Plasma tumor necrosis factor-*alpha* (TNF-α; A) and interleukin-1 *beta* (IL-1β; B) of mid-lactating Fleckvieh cows fed either a standard diet (CTR) or diet supplemented with rumen-protected dry grape extract at 470 mg/head/day (NG-BPO) for 35 d during a natural heat stress exposure in summer.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

5.4.5. Hematology Response

The hematology response of heat-stressed dairy cows supplemented or not with Nor-Grape BPO is reported in Table 6.

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

	Treatment[1]			***P*-value**[3]		
Item	**CTR**	**NG-BPO**	**SEM**[2]	**Trt**	**Time**	**Trt * Time**
Red blood cells, x 10^6/μL	6.12	6.13	0.18	0.95	< 0.01	0.59
White blood cells, x 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
Platelet, x 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
Platelet crit, %	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
Lymphocytes count, x 10^6/mL	4.43	4.79	0.37	0.17	0.06	0.28
Monocytes count, x 10^6/mL	0.15	0.14	0.02	0.44	< 0.01	0.50
Neutrophils count, x 10^6/mL	3.60	3.18	1.17	0.55	0.99	0.32
Eosinophils count, x 10^6/mL	0.41	0.31	0.08	0.05	0.11	0.05
Basophils count, x 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/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] Greatest standard error of the mean.

[3] 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.

The amount of platelet was overall lower in NG-BPO cows (Trt; *P* = 0.03) compared with CTR cows, with also an interaction effect (Trt * Time; *P* = 0.03), being lower at 17 d of treatment

(NG-BPO: 405.63 *vs.* CTR: 524.21 ± 27.10 cells x 10^6/mL; *P* = 0.001). Overall, the eosinophils percentage was significantly lower in the NG-BPO group compared with CTR group (Trt; *P* = 0.03), with also an 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 the 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 lower circulating eosinophil cells at 35 d of treatment (NG-BPO: 0.30 *vs.* CTR: 0.52 ± 0.06 cells x 10^6/mL; *P* = 0.05).

5.4.6. Phagocytosis

Results of neutrophils and monocytes phagocytosis activity are shown in Figure 6. In detail, the 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; Fig. 6A) as well as neutrophil phagocytosis activity (NG-BPO: 39.32 *vs.* CTR: 32.08 ± 2.32 %; Trt: *P* = 0.03; Fig. 6B).

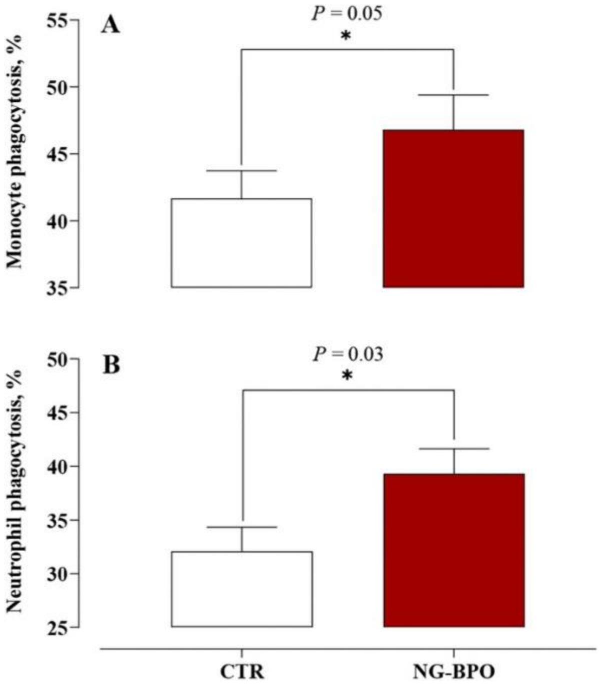

Figure 6. Monocytes (A) and neutrophils (B) phagocytosis activity against *E. coli* at 35 d of treatment of mid-lactating Fleckvieh cows fed either a standard diet (CTR) or diet supplemented with rumen-protected dry grape extract at 470 mg/head/day (NG-BPO) for 35 d during a natural heat stress exposure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

5.4.7. Rumen Fluid Data

The effects of dietary supplementation of Nor-Grape® BP-O on rumen fluid pH, lactates, ammonia and volatile fatty acids (VFAs) are shown in Table 7.

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 (NG-BPO) for 35 d during a natural heat stress exposure in summer.

	Treatment[1]			***P*-value**[3]		
Item	**CTR**	**NG-BPO**	**SEM**[2]	**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
Valeriate	0.88	0.89	0.04	0.67	0.01	0.49
Isovaleriate	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
Valeriate	0.95	0.97	0.24	0.24	< 0.01	0.89
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/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] Greatest standard error of the mean.

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

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 observed also 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 approximately 74 on average to approximately 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).

5.5. Discussion

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

The use of dietary supplements during HS has the main objective to reduce 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 help to improve not only the oxidative status, but also support the 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 effect could be more efficient, by-passing rumen degradability and being absorbed by the small intestine; in fact, previous studies demonstrated that grape polyphenol’s antioxidant activity is degraded in ruminal fluid (Chedea et al., 2016). Furthermore, previous studies 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 milk yield (MY), it indicated that the NG-BPO group had a higher THI breakpoint compared with CTR group. The data indicates also 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 keep a higher level of milk yield 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 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 polyphenols amount 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 days, whereas in the study by Gessner et al. (2015), the amount of polyphenols/cow/day, was about 6,240 mg for the dry cows and 11,440 mg for the lactating cows for 90 days. 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 polyphenols content was not specified, as well as 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 approximately 32,540 mg of polyphenols/cow/day, showing any significant differences in milk production and quality compared to cows without supplementation. On the other hand, Nudda et al. (2019), reported an increase in MY in lactating ewes fed with grape marc, where the amount of polyphenols per head was about 1,480 mg per day. The authors suggested that the polyphenols may have interacted with proteins forming complexes, and thus reducing protein solubility, 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 higher milk production (Patra and Saxena, 2011). This result is also supported by the greater ECM and FCM showed by the NG-BPO group compared to the CTR group, and by the greater content in milk yield fat and protein. Moreover, the greater milk production and FCM content may be also supported by the lower rectal temperature 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).

Regarding the SCC, it 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 content with a supplementation of 100 g of grape per ewe per day, providing 1,480 mg polyphenols/ewe/day.

Furthermore, Huang et al. (2019) did not observe differences either in SCC after supplementing up to 80 mg of a grape seed extract/kg of body weight/day containing 95% procyanidins, equivalent to supplementing 53,200mg of non-protected polyphenols/cow/day for a 700 kg cow. However, in the present study cows supplemented with NG-BPO (equivalent to 282 mg protected polyphenols/cow/day) exhibited a decrease in SCC compared to the CTR group, suggesting a better immunostimulatory response and a healthier mammary gland even with lower polyphenols amount, 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 the 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. The increase in respiration rate is one of the primary cooling mechanism in dairy cows for heat load, which can reach up to 200 breaths per minute during a HS condition (Mader et al., 2006). On the other hand, 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 a significantly greater HB compared to NG-BPO group, especially during the last week of the trial and in correspondence with the heat wave. Thus, can be affirmed that whereas the CTR group experienced a condition of HS, the 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 CTR group ($R^2 = 0.88$) compared to NG-BPO cows ($R^2 = 0.70$), and the significantly stronger correlation coefficient in CTR cows between the two 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 to 4.78 min/d of the NG-BPO group, for each unit of THI above the threshold, suggest a better ability to cope with HS with a more moderate increase of HB in NG-BPO group with rising THI.

Regarding the trend of RT, it was different among the groups, especially during the morning. Indeed, the NG-BPO group exhibited lower RT at 17, 28 and 35 days of treatment. This result showed how the NG-BPO group was more able to dissipate heat, maintaining lower the body temperature, although cows had to support a greater milk production compared to the CTR cows. During HS, the occurring of oxidative stress involves in high production of free radicals and oxygen reactive species, damaging cells and tissues and also affecting as consequence the immune function (Bernabucci et al., 2002). In this context, the polyphenols supplementation had shown to exert anti-inflammatory effects during HS when supplemented as feed additives (Gessner et al., 2017). Although in this study no significant differences between groups were observed in biomarkers of oxidative stress (ROMt, SHp, FRAP and AOPP), it is noteworty that polyphenols also have a wider effect on the organism. A recent studies on pullets (Aberkane et al., 2025) have demonstrated that polyphenols can exhert pleiotropic effects on animal physiology, extending beyond their well-known antioxidant capacity. Indeed, in this study the supplementation of Nor-Grape showed significant effects on early skeletal development and modulation of metabolomic pathways of pullets, improving bone mineralization and strength, reducing the prevalence of bone defects. Therefore, the improved thermoregulation and immune response observed in this trial may result from polyphenol-driven metabolic and cellular adaptations, rather than (or in addition to) direct oxidative stress mitigation.

Herein, haptoglobin resulted lower in NG-BPO cows compared to CTR cows after 31 days of treatment. Haptoglobin is the main positive acute phase protein in cows, which occurs during an inflammatory status and oxidative stress (Bertoni and Trevisi, 2013), and higher levels were previously associated to an HS condition (Maggiolino et al., 2025). From the results of this study, the polyphenols availability in Nor-Grape® BP-O may have supported and regulated inflammation of dairy cows during a HS condition, contrasting blood haptoglobin levels. While 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 days, when a 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 a rise in THI and the occurrence of a heat wave. This response might indicate an 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 a differential modulation of the immune system. In fact, the presence of polyphenols can modulate immune response in both the innate and adaptive systems, having both stimulatory and inhibitory effects (Hachimura et al., 2018).

The better inflammatory response is confirmed also 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 results lower during severe inflammatory condition. Previous studies (Amato et al., 2024) reported lower blood haptoglobin and a greater Zn concentration in cows after supplementation of olive cake rich in polyphenols (approximately 560.32 mg polyphenols/cow/day for 30 days), as an effect to improve the liver function and at the same time to induce a less pronounced inflammatory response. Polyphenols are known for their antioxidant, anti-radical and radical scavenging activities, and previous studies also indicates 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 had previously reported a decrease in plasma glucose in cows under HS, which mainly arise from low DMI, and higher levels of plasma insulin (Wheelock et al., 2010). The alteration of the feed intake during HS and the consequent negative energy balance can also alter the carbohydrate, lipid and protein metabolism (Calamari et al., 2011). Although in this study there were 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 globulin are indicators of cow’s immune response, and they include also immunoglobulins (Bionaz et al., 2007). Indeed, immunoglobulins (Ig) are functionally and structurally differentiated class of globulins that act as antibodies. Most of Ig belong to the class of IgG, with an important role protecting 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 timepoint of the treatment (17, 31 and 35 days), could be supportive of a greater Ig production and thus a better adaptive immune response of NG-BPO cows. As support of this speculation, the study of Engler et al. (2022) demonstrated that 670 mg Nor-Grape® BP-O/cow/day increases the humoral response thanks to the presence of polyphenols (Shakoor et al., 2021), and this evidence was 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 days of treatment, compared to the CTR group. Although the decrease of A:G ratio is associated to an 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) the A:G could not be related to an inflammatory response, as its value is in the physiological range (Bionaz et al., 2007), but, probably, the lower ratio in NG-BPO cows could be associated to a lower dehydration (lower albumin levels) and to a stimulation of humoral response (greater globulins).

It is noteworthy, that during HS the liver function is altered, caused by nutritional and metabolic alteration (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 a better hepatic function, as consequence of a better excretory capacity of the liver. The hypothesis of a better liver functionality is supported by results of Gessner et al. (2015), which observed, after supplementing dairy cows with a polyphenol-rich extract derived from grape, a significant reduction in the mRNA expression of genes associated with inflammation and endoplasmatic reticulum stress in the liver. Interestingly, this improvement, in the present study, was obtained with only 282 mg rumen-protected polyphenols/cow/day, compared to 11,440 mg unprotected polyphenols/cow/day in Gessner et al. (2015) study (22kg of DMI/cow/day, containing 1% of grape product at 52 mg polyphenols/g).

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

Previous studies documented a reduction of the immune response in heat-stressed dairy cows associated to a lower production of TNF-α (Lacetera et al., 2005; Caroprese et al., 2009). At 17 days of treatment TNF-α levels were initially lower in NG-BPO group, but then after 31 days of supplementation they increased. As reported by Lendez et al. (2021) the diminishment of this cytokine during HS makes the animals not physiologically prepared to resist high temperatures, having a less efficient immune response and, hence, making them more susceptible to opportunistic infections. This finding was supported also by Safa et al. (2019), which 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 a dysregulation of inflammatory responses (Safa et al., 2019) in this group, which, on the other hand, was well supported by its higher production in NG-BPO group. Indeed, TNF-α has an important role in stimulating the phagocytosis activity of neutrophils and in mobilize macrophages and neutrophils to the site of infection (Whiteside, 2007).

Interleukin-1β (IL-1β) is a pro-inflammatory cytokine which through the stimulation of diapedeses and chemoattraction of neutrophils and monocytes, is also important to promote phagocytosis (Peker and Musal, 2022). In an in vitro HS-induced study, isolated polymorphonuclear leukocytes 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 the cells' ability to effectively counteract pathogenic challenges. Therefore, the greater concentration of this pro-inflammatory 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 the milk synthesis (Bradford et al., 2015).

Since NG-BPO cows exhibited lower panting and RT, they likely experienced a less physiological pressure, which probably contributed to maintain lower circulating blood cortisol. Although in this study blood cortisol was not measured, the greater IL-1β and TNF- α could be indicative of a reduced cortisol secretion, since 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 a better immune function during naturally occurring HS in those cows and also a more efficient resolution of the systemic inflammatory response, as supported by the lower haptoglobin, especially during the occurrence of an "heat waves". This can potentially support cytokine functionality and thus reducing the negative immunosuppressive effects typically observed under HS conditions.

Taken togheter, this response can indicate an enhancement of the innate and adaptive immune functions, helping NG-BPO cows to maintain a more effective immune response. Indeed, this affirmation is confirmed by a better phagocytosis activity in NG-BPO group compared to CTR group, showed by greater phagocytosis activity of both neutrophils and monocytes.

The variation of season and thus environmental temperature influence also hematological parameters. During HS, hematological alteration can be related with disease (Park et al., 2021) or to the high consumption of water usually detected with the rising temperature, which occurs in hemodilution (Casella et al., 2013). Although in this study we did not observe significant differences between groups on these blood parameters, platelets, eosinophils and eosinophils count were lower in NG-BPO cows. Unexpectedly, in our study and in contrast with these previous studies, the supplementation of rumen-protected grape extract was associated with a decrease in blood platelets. However, studies in mices have shown that a stress condition enhance the increase of platelet activity, since after stress exposition there is the sympathetic stimulation of norepinephrine and epinephrine which 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), which observed a significant increase in Holstein steers under HS, suggesting the susceptibility of these immune related cells to HS, and suggesting to consider 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 two 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.

5.6. Conclusions

In conclusion, the dietary inclusion of rumen-protected grape polyphenols offers a promising nutritional strategy to mitigate the adverse effects of naturally ocuirring heat stress 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 modulation, and inflammatory regulation highlight its potential as an effective and practical tool to promote resilience and productivity in heat-stressed dairy herds.

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CHAPTER 6

Final Considerations

Studying the response to climate change in autochthonous cattle breed is crucial to understand and objectively characterize their ability to cope with HS, in particular considering that these breeds are mainly reared under semi-extensive systems. In such conditions, dairy cows are subjected to hard challenge for their resilience, especially in the Mediterranean regions, where climate changes are particularly impacting the region.

Although local breeds are often assumed to be more resilient than cosmopolitan breeds (being able to live in harsh environment with poor quality feed) such as Holstein dairy cow, it is important to objectively define their degree of thermoresilience. In this context, the findings of this thesis provide an integrated assessment of HS responses in Sicilian local cattle breeds, highlighting their potential role within livestock systems in Sicily and supporting their valorisation.

Indeed, the valorisation of innate adaptive traits may represent a viable pathway toward more sustainable and economically efficient product systems, with potential applicability to comparable farming systems in Mediterranean regions.

Overall, the results of this thesis confirm that autochthonous breeds have intrinsic thermotolerance, as showed by the ability of Cinisara cow to maintain thermal stability and productive performance under elevated THI conditions, indicating an efficient adaptive response.

Similar findings were observed in Modicana cows, in which animals were also able to maintain stable milk production and quality during HS. In addition, in this study was also investigated oxidative and metabolic status, with the main evidence that also local breed can be negatively impacted by HS, with signs of oxidative imbalance. For this reason, it is important to characterize breed-specific adaptive strategies in order to identify potential target management approaches.

In general, the lower milk production (and therefore lower metabolic heat) in Cinisara and Modicana compared with other cosmopolitan breeds, may contribute to reduce the sensibility to HS. Moreover, the objectively assessed adaptive ability, likely linked by their genetic background represent a key element for their conservation and continued use, in a context where local breeds are increasingly being replaced by more productive cosmopolitan breeds. Thus, the findings of this thesis support the valorisation of Sicilian autochthonous cattle breeds within climate-resilient, sustainable, and economically efficient farming systems.

This thesis also contributes to the use of nutritional supplementation as a viable strategy to mitigate HS in dairy cows. Indeed, the positive effects observed with rumen-protected grape polyphenols indicate that the modulation of oxidative and immunometabolic response, can help maintain physiological homeostasis, animal behaviour and production during a natural HS exposure. Moreover, because the polyphenols were rumen-protected, their effects may be enhanced by bypassing ruminal degradation and allowing absorption at the intestinal level. These findings highlight the potential of nutritional interventions to enhance physiological resilience under challenging environmental conditions (especially during summer), offering a flexible and potentially cost-effective approach that can be integrated with existing management practices.

Taken together, as climate challenges intensify, integrating genetic, physiological, and managerial perspectives will be essential to sustain productivity, animal welfare, and for the preservation of livestock farming in Mediterranean regions. Specifically, investigating the genetic basis of thermoresilience would be an important baseline to further characterize local breeds and support their conservation and selection.

Conservation strategies aim at developing and maintaining livestock populations that are well adapted to specific agro-climatic zones, combining thermotolerance, drought resistance (increasingly frequent), and the ability to thrive in low-input systems.

For this reason, further study is necessary to estimate the genetic component of heat tolerance in Sicilian cattle, considering that the correct estimation of THI thresholds represents the first step to identify components that could be included in selection procedures.

These activities could also include the investigation of biological mechanisms in order to distinguish adaptive responses associated with lower production efficiency (and consequently reduced metabolic heat load) from those intrinsically linked to breed-specific genetic background. In this regard, integrated approaches combining phenotypic indicators, physiological biomarkers, and functional genomics tools may help to identify key biological pathways and genomic profiles involved in thermoregulation and heat resilience. A deeper understanding of these mechanisms would allow a more objective characterization of breed-specific adaptive capacity. For example, the application of advanced genomic approaches, including quantitative trait loci (QTL) mapping, genome-wide association studies (GWAS), and transcriptomic profiling under HS conditions, to better elucidate the genetic architecture underlying thermotolerance in Sicilian cattle breeds.

Moreover, the identification of molecular markers (e.g. through gene expression profiling), and regulatory pathways associated with thermotolerance, could have relevant translational implications, supporting breeding strategies aimed at improving climate resilience also in other dairy breeds.

APPENDIX A
List of Publications

Journal Publications:

1. Fazio Esterina, Bionda Arianna, Liotta Luigi, **Amato Annalisa**, Chiofalo Vincenzo, Crepaldi Paola, Satué Katiuska, and Lopreiato Vincenzo (2022). Changes of acute-phase proteins, glucose, and lipid metabolism during pregnancy in lactating dairy cows. Archives Animal Breeding, 65, 329–339. doi: 10.5194/aab-65-329-2022.

2. Bionda Arianna, Lopreiato Vincenzo, Crepaldi Paola, Chiofalo Vincenzo, Fazio Esterina, Oteri Marianna, **Amato Annalisa** and Liotta Luigi. Diet supplemented with olive cake as a model of circular economy: Metabolic and endocrine responses of beef cattle - In: frontiers in sustainable food systems. - ISSN 2571-581X. - 6:(2022 Dec 20), pp. 1077363.1-1077363.14. [10.3389/fsufs.2022.1077363]

3. **Amato Annalisa,** Minuti Andrea, Liotta Luigi, Cattaneo Luca, Sfulcini Marta, Trevisi Erminio, & Lopreiato Vincenzo (2023). Whole cottonseed inclusion in starter feeds improves performance, inflammometabolic profile, and rumination behavior in Holstein dairy calves. JDS communications, 4(5), 422-427. https://doi.org/10.3168/jdsc.2022-0368

4. Floridia Viviana, Russo Nunziatina, D'Alessandro Enrico, Lopreiato Vincenzo, Pino Alessandra, **Amato Annalisa**, Liotta Luigi, Caggia Cinzia and Randazzo Cinzia Lucia (2023). Effect of olive cake supplementation on faecal microbiota profile of Holstein and Modicana dairy cattle. Microbiological Research, 277, 127510. https://doi.org/10.1016/j.micres.2023.127510

5. Bionda Arianna, Lopreiato Vincenzo, Amato Annalisa, Cortellari Matteo, Cavallo Carmelo, Chiofalo Vincenzo, Crepaldi Paola, and Liotta Luigi (2023). Phenotypic and Genomic Characterization of the Comune di Sicilia Goat: Towards the Conservation of an Endangered Local Breed. Animals, 13(20), 3207.

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6. Ferronato Giulia, Cattaneo Luca, **Amato Annalisa**, Minuti Andrea, Loor Juan J., Trevisi Erminio, Cavallo Carmelo, Attard George, Elolimy Ahmed A., Liotta Luigi and Lopreiato Vincenzo (2023). Residual feed intake is related to metabolic and inflammatory response during the preweaning period in Italian Simmental calves. J Dairy Sci. 2024 Mar;107(3):1685-1693. doi: 10.3168/jds.2023-23617. Epub 2023 Nov 8. PMID: 37944812.

7. **Amato Annalisa**, Cavallo Carmelo, Marín-García, Pablo Jèsus, Emmanuele Giovanni, Tomasello, Mario, Tomasella Cristina, Floridia Viviana, and Llobat Lola (2024). Effect of breed on hematological and biochemical parameters of apparently healthy dogs infected with zoonotic pathogens endemic to the Mediterranean Basin. Animals, 14(11), 1516. https://doi.org/10.3390/ani14111516

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9. Litrenta, F., Cincotta, F., Russo, N., Cavallo, C., Caggia, C., **Amato, A**., Lopreiato, V., Merlino, M., Verzera, A., Randazzo C.L., Liotta, L. (2024). Feeding Cows with Olive Cake Enriched in Polyphenols Improves the Sustainability and Enhances the Nutritional and Organoleptic Features of Fresh Caciocavallo Cheese. *Foods*, *13*(20), 3320. doi: https://doi.org/10.3390/ani14223336

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12. **Amato, A**., Cavallo, C., Marín-García, P. J., Emmanuele, G., Tomasello, M., Tomasella, C., Floridia V., Liotta L., Llobat, L. (2024). Effect of breed on hematological and biochemical parameters of apparently healthy dogs infected with zoonotic pathogens endemic to the Mediterranean Basin. *Animals*, *14*(11), 1516. doi: https://doi.org/10.3390/ani14111516

13. Alberghina, D., **Amato, A**., Brancato, G., Cavallo, C., Liotta, L., & Lopreiato, V. (2024). Impact of Heat Stress on the Balance between Oxidative Markers and the Antioxidant Defence System in the Plasma of Mid-Lactating Modicana Dairy Cows. *Animals*, *14*(14), 2034. doi: https://doi.org/10.3390/ani14142034

14. Cavallo, C., **Amato, A**., Liotta, L., Minuti, M., Lopreiato, L. The adaptation in the first week to the automatic milking system influences long-term milk performance and behavior of Holstein cows. Slovak Journal of Animal Science, October 2024. https://doi.org/10.36547/sjas.952

15. Lopreiato, V., Ferronato, G., **Amato, A**., Cavallo, C., Trevisi, E., LIobat, L., Chiofalo, V., Liotta, L. (2025). Effects of dietary supplementation with olive cake enriched in polyphenols on growth, rumen fermentation, and metabolic status of finishing Limousine bulls. Italian Journal of Animal Science, 24(1), 174-181. https://doi.org/10.1080/1828051X.2024.2445165

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18. **Amato, A**., Cavallo, C., Minuti, A., Trevisi, E., Engler, P., Bui, H., Gugliandolo, E., Llobat, L., Laporta, J., Emmanuele, G., Liotta, L., & Lopreiato, V. (2025). Rumen-protected dry grape extract supplementation enhances milk production, behavior traits, and immunometabolism of mid-lactating Fleckvieh cows under naturally occurring heat stress. Journal of Dairy Science. https://doi.org/10.3168/jds.2025-26830

19. Floridia, V., Bionda, A., Arias, K. D., **Amato, A**., Cavallo, C., Chiofalo, V., Cortellari, M., Lopreiato, V., Virga, A.N., Crepaldi, P., Barbato, M. & Liotta, L. (2025). Genomic insights on the history and selection trajectories of the Comune di Sicilia goat. BMC Genomic Data. https://doi.org/10.1186/s12863-025-01368-5

20. Carmelo Cavallo, **Annalisa Amato**, Luigi Liotta, and Vincenzo Lopreiato. Estimation of heat stress thresholds in Sicilian Cinisara lactating cows under naturally occurring conditions. *JDS communications*. https://doi.org/10.3168/jdsc.2025-0797

21. Martínez-Sáez, L., Marín-García, P. J., **Amato, A.,** Cavallo, C., Boccuni, S., Tomasello, M., Liotta, L., & Llobat, L. (2025). Breed-specific variation in canine cytokine profiles in a leishmaniosis-endemic region. Microbial Pathogenesis, 108096. https://doi.org/10.1016/j.micpath.2025.108096

22. Cavallo, C., **Amato, A**., Lopreiato, V., Paparone, N., Scalone, D., Litrenta, F., & Liotta, L. (2025). Improving Sustainability in Buffalo Finishing: Olive Cake Supplementation and Its Effects on Performance and Meat Quality. Applied

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Conference presentations:

23. **Annalisa Amato**, Luigi Liotta, Carmelo Cavallo, Vincenzo Chiofalo, Esterina Fazio, Arianna Bionda, Vincenzo Lopreiato. (2022). Effects of olive cake inclusion in the diet of Modicana dairy cows on milk quality. 75° Congresso Nazionale SISVET, 15–18/06/2022, Lodi (MI). ISBN 978-88-909092-3-8

24. Francesca Accetta, Luigi Liotta, **Annalisa Amato**, Rosita La Cava, Doriana Aliquò, Cinzia Randazzo, Ambra Rita Di Rosa. (2022). E-nose and E-tongue for sensory quality evaluation of canestrato cheese produced with kiwifruit extract**.** 75° Congresso Nazionale SISVET, 15–18/06/2022, Lodi (MI). ISBN 978-88-909092-3-8

25. Abdulrahman Alharthi, Giulia Ferronato, Andrea Minuti, Ahmed Elolimy, Fiorenzo Piccioli-Cappelli,Erminio Trevisi, **Annalisa Amato**, Luigi Liotta and Vincenzo Lopreiato. (2022). Residual feed intake and its association with blood inflammometabolic profile in preweaning Simmental calves. 75° Congresso Nazionale SISVET, 15–18/06/2022, Lodi (MI). ISBN 978-88-909092-3-8

26. Lopreiato Vincenzo, Bionda Arianna, **Amato Annalisa**, Fazio Esterina, Crepaldi Paola, Chiofalo Vincenzo, and Liotta Luigi. Dietary olive cake inclusion and metabolic change of lactating Holstein, Simmental and Modicana cows. In Book of Abstracts of the 73rd Annual Meeting of the European Federation of Animal Science (2022), 264. Wageningen Academic Publishers. doi: 10.3920/978-90-8686-937-4 [Poster presentato a: 73rd Annual Meeting of EAAP, Porto, 5-9 settembre 2022].

27. Federica Litrenta, Arianna Bionda, Vincenzo Lo Turco, Angela Giorgia Potortì, Luigi Liotta, **Annalisa Amato**, Giuseppa Di Bella. (2022). Bisphenol residues in conventional and unconventional provolas cheese. In J. Pulkrabová, M. Tomaniová, S. van Leeuwen, M. Nielen, & J. Hajšlová, Book

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28. Federica Litrenta, Luigi Liotta, Vincenzo Lopreiato, **Annalisa Amato**, Angela Giorgia Potortì, Giuseppa Di Bella. Effect of olive cake on the mineral profile of Ragusan provola cheese. XIII congresso nazionale di chimica degli alimenti. ISBN 978-88-94952-37-7

29. **Annalisa Amato**, Sonia Bonacci, Marianna Oteri, Carmelo Cavallo, Vincenzo Lopreiato, Luigi Liotta. Milk Fatty Acids profile in Modicana and Holstein cows under the same feeding management. XIII congresso nazionale di chimica degli alimenti. ISBN 978-88-94952-37-7

30. Luigi Liotta, Vincenzo Lopreiato, **Annalisa Amato**, Maria Elena Furfaro, Monica Greco, Nirey Sirio Velez Cervera, Andrea Letizia Bonsignore, Rossana Denaro, Mirela Raluca Folea, Carmelo Cavallo, Cristina Tomasella, Vincenzo Chiofalo. Chemical artisanal cheese characterization from Comune di Sicilia goat ecotype. XIII congresso nazionale di chimica degli alimenti. ISBN 978-88-94952-37-7

31. **Annalisa Amato**, Marianna Oteri, Carmelo Cavallo, Sonia Bonacci, Vincenzo Chiofalo, Luigi Liotta. Evaluation of fatty acids profile of milk produced by mid-lactating Holstein cows fed with enriched olive cake diet. Pasquale De Palo (2023) ASPA 25th Congress Book of Abstract, Italian Journal of Animal Science, 22:sup1, 1-320, DOI: 10.1080/1828051X.2023.2210877

32. Vincenzo Lopreiato, Federica Litrenta, Giuseppa Di Bella, **Annalisa Amato**, Carmelo Cavallo, Luigi Liotta, Vincenzo Chiofalo. Circular bioeconomy for olive oil waste and by-product valorization: Effect of dietary enriched olive cake inclusion in Modicana cows feeding management on cheese quality. Pasquale De Palo (2023) ASPA 25th Congress Book of Abstract, Italian Journal of Animal Science, 22:sup1, 1-320, DOI: 10.1080/1828051X.2023.2210877

33. Luigi Liotta, Vincenzo Lopreiato, **Annalisa Amato**, Carmelo Cavallo, Cristina Tomasella, Arianna Bionda, Marco Scalisi and Vincenzo Chiofalo. Phenotypic characterization of an endangered indigenous goat: Comune di Sicilia ecotype. Pasquale De Palo (2023) ASPA 25th Congress Book of Abstract, Italian Journal of Animal Science, 22:sup1, 1-320, DOI:10.1080/1828051X.2023.2210877

34. Vincenzo Chiofalo, Vincenzo Lopreiato, **Annalisa Amato**, Carmelo Cavallo, Marco Scalisi, Viviana Floridia and Luigi Liotta. Morphological description of Montanina cattle, an old Sicilian (Italy) indigenous ecotype. Pasquale De Palo (2023) ASPA 25th Congress Book of Abstract, Italian Journal of Animal Science, 22:sup1, 1-320, DOI:10.1080/1828051X.2023.2210877.

35. Lola Llobat, **Annalisa Amato**, Mario Tomasello, Carmelo Cavallo, Viviana Floridia, Cristina Tomasella, Luigi Liotta. Coinfections of Leishmania infantum and other zoonotic pathogens in apparently healthy Cirneco dell'Etna dogs' breed. Screening of hematological traits. ALIVE 2, Animal Leishmaniosis International Veterinary Event, 18-20 aprile 2024, Nizza, Francia.

36. Rumen fermentation parameters prediction model using mid-infrared spectra of rumen fluid collected from Simmental dairy cows. C. Cavallo, **A. Amato**, A. Minuti, V. Lopreiato, M. Tolone. 75th Annual Meeting of the European Federation of Animal Science, 01-05 settembre 2024, Firenze, Italy. ISBN: 979-12-210-6769-9

37. Rumen-protected dry grape extract supply during natural heat stress improves the whole-blood immune response of Simmental cows. M. Forleo, **A. Amato**, C. Cavallo, E. Gugliandolo, L. Liotta, E. Trevisi, A. Minuti, H. Bui, P. Engler, V. Lopreiato. 75th Annual Meeting of the European Federation of Animal Science, 01-05 settembre 2024, Firenze, Italy. ISBN: 979-12-210-6769-9

38. Supplemental rumen-protected dry grape extract alters the metabolic response and rumen fermentation of Simmental cows during heat stress. **A. Amato**, M. Forleo,

C. Cavallo, L. Liotta, E. Trevisi, A. Minuti, H. Bui, P. Engler, V. Lopreiato. 75th Annual Meeting of the European Federation of Animal Science, 01-05 settembre 2024, Firenze, Italy. ISBN: 979-12-210-6769-9

39. Effects of dietary supplementation with enriched olive cake on growth, rumen fermentation, and metabolic status in Limousine bulls. C. Cavallo, **A. Amato**, G. Ferronato, V. Lopreiato, L. Liotta. 75th Annual Meeting of the European Federation of Animal Science, 01-05 settembre 2024, Firenze, Italy. ISBN: 979-12-210-6769-9

40. Effect of CLA-producing adjunct cultures of nutritional value of sheep cheese. Litrenta, Federica; Bosco, Georgiana; **Amato, Annalisa**; Cavallo, Carmelo; Scalisi, Marco; Lucia Randazzo, Cinzia; Nava, Vincenzo; Lopreiato, Vincenzo; Liotta, Luigi. 11th International Symposium on Recent Advances in Food Analysis, September 5–8, 2024 - Prague, Czech Republic. ISBN 978-80-7592-268-7

41. Utilizzo di una coltura starter nella caseificazione del latte ovino per incrementare il contenuto di CLA. Bosco Georgiana, Litrenta Federica, Randazzo Cinzia Luigia, Liotta Luigi, **Amato Annalisa**, Cavallo Carmelo, Scalisi marco, Lopreiato Vincenzo. XXV CONGRESSO SIPAOC, Società Italiana di Patologia e di Allevamento degli Ovini e dei Caprini. SASSARI 17-20 SETTEMBRE 2024.

42. Temperature-humidity index breakpoints for rectal temperature and milk performance of Cinisara cows under semi-extensive dairy system. Carmelo Cavallo, **Annalisa Amato**, Carola Evola, Riccardo Negrini, Marco Tolone, Luigi Liotta, Vincenzo Lopreiato. ASPA 26th Congress, 17-20 June 2025, Turin.

43. The impact of pre-weaning plane of nutrition on mammary gland development of dairy calves. **Annalisa Amato**, Marta Sfulcini, Maverick Guenther, Grace Larsen, Caroline Guzi Savegnano, Sha Tao, Jimena Laporta. ASPA 26th Congress, 17-20 June 2025, Turin.

44. Uncovering the architecture of production-driven introgression in Cinisara cattle. Viviana Floridia, Katherine Daniela Arias, Arianna Bionda, Matteo Cortellari, **Annalisa Amato**, Vincenzo Lopreiato, Enrico D'alessandro, Felix Goyache, Paola Crepaldi, Luigi Liotta, Mario Barbato. ASPA 26th Congress, 17-20 June 2025, Turin.

45. iSafe-Graze: Selection of Probiotic Lactic Acid Bacteria as Starter Cul-tures to Enhance Conjugated Linoleic Acid Content in Sheep's Milk Cheese. Carmelo Cavallo, Vincenzo Lopreiato, Georgiana Bosco, Federica Litrenta, Cinzia Randazzo, Marco Scalisi, **Annalisa Amato**, Viviana Floridia, Luigi Liotta. ASPA 26th Congress, 17-20 June 2025, Turin.

46. Mapping the phenotypic and genomic landscape of Montanina cattle. Viviana Floridia, Arianna Bionda, Vincenzo Chiofalo, Matteo Cortellari, Vincenzo Lopreiato, **Annalisa Amato**, Antonino Nazareno Virga, Paola Crepaldi, Luigi Liotta, Mario Barbato, Viviana Floridia. ASPA 26th Congress, 17-20 June 2025, Turin.

47. Breed-specific hematological phenotypes in dogs apparently healthy but infected by zoonotic pathogens: the case of Fonnese and Cirneco dog breeds. Lola Llobat, Sara Sechi, Luigi Liotta, **Annalisa Amato**, Carmelo Cavallo, Raffaella Cocco. ASPA 26th Congress, 17-20 June 2025, Turin.

48. Effect of extruded linseed supplementation on milk quality, fatty acids profile, and rennet-induced coagulation properties in mid-lactating ewes. **Amato Annalisa**, Lopreiato Vincenzo, Cavallo Carmelo, Lunetta Maria, Scalisi Marco, Litrenta Federica, Liotta Luigi.78°Convegno SISVET, 10-12 Giugno 2025, Giardini Naxos.

49. Maverick C. Guenther, **Annalisa Amato**, Marta Sfulcini, Grace A. Larsen, Caroline G. Savegnago, Adam Geiger, Sha Tao, and Jimena Laporta. The impact of pre-weaning plane of nutrition and heat abatement on mammary gland development of dairy calves. 2025 American Dairy Science Association Annual

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50. Savegnago C.G, Y.B. Amancio, J. Gao, A. M. Roper, T.N. Marins, N. Andriotti, M.C. Guenther, **A. Amato**, J. Laporta, A.J. Geiger, and S. Tao. Impacts of plane of nutrition and heat abatement during the preweaning period on growth of dairy heifers. 2025 American Dairy Science Association Annual Meeting: Louisville, Kentucky.

51. Saha C., C.G. Savegnago, Y.B. Amancio, R.M. da Costa, J. Gao1, A.M. Roper, T. N. Marins, N. Andriotti, M.C. Guenther, **A. Amato**, N. J. Laporta, S. Tao, A.A.C. Alves Computer Vision-Tracked Behavior in Dairy Calves 2025 American Dairy Science Association Annual Meeting; Louisville, Kentucky.

52. Heavy breathing by accelerometer-based recordings is associated with body temperature, rumen and blood metabolites of Fleckvieh dairy cows during heat stress: A cluster-analytic approach. **A. Amato**, C. Cavallo, E. Trevisi, A. Minuti, M. Ghaffari, L. Liotta, and V. Lopreiato. The 76th Annual Meeting 25/29 August 2025 – Innsbruck, Austria.