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Signals in the Milk:
What Melatonin and MiRNAs Reveal About Mastitis in Dairy Cows

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Alla mia famiglia,
umana e a quattro zampe,
che mi accompagna in ogni passo

"La grandezza di una nazione e il suo progresso morale si possono giudicare dal modo in cui tratta gli animali." – Mahatma Gandhi

Summary

Abstract

Bovine mastitis is one of the most common and economically significant diseases in the dairy industry, creating the need for novel, non-invasive diagnostic tools and alternative strategies to control udder health. This thesis investigated two aspects of the mammary gland immune response, with the main aim of identifying bioactive molecule mediators and molecular biomarkers that could enhance, respectively, mastitis prevention and monitoring.

The first part of the study (experiment 1) focused on determining melatonin concentrations in bovine milk collected during daytime and nighttime milkings in winter, under natural photoperiod conditions. Milk samples were collected from 40 Holstein Friesian cows at 4:00 am and 4:00 pm and analysed for composition, melatonin content, somatic cell count (SCC), and differential somatic cell count (DSCC). Melatonin quantification was performed with an ELISA kit, while SCC and DSCC were determined with a Fossomatic™ 7DC analyser. Melatonin concentrations were significantly higher in nighttime milk samples (15.63 ± 1.9 pg/mL *vs.* Day group: 6.80 ± 0.75 pg/mL) ($p < 0.001$). Furthermore, Night milk samples showed a SCC reduction (129.70 ± 25.01 $\times 10^3$ cells/mL *vs.* Day group: 196.39 ± 33.80 $\times 10^3$ cells/mL) ($p < 0.001$), confirming the hypothesis that melatonin may play a protective and immunomodulatory role on udder health. DSCC showed no differences between Day and Night samples ($p = 0.78$), indicating that the proportion of immune cell subpopulations does not vary during the day and that melatonin may act by enhancing

the local immune response rather than altering its composition. These results highlight melatonin potential as a natural bioactive molecule that contributes to mammary gland defence and can be modulated through improved livestock management.

The second part of the study (experiment 2) evaluated the potential as biomarkers for mastitis early diagnosis of four circulating microRNAs (miR-26a-5p, miR-142-5p, miR-146a, and miR-223-3p) in milk, already associated with mammary gland inflammation. The study assessed the c-miRNAs quantification by qPCR both *in vivo* in milk samples grouped by inflammatory status (healthy, susceptible, chronic, and acute mastitis), and *in vitro* in the inflammatory-stimulated supernatants of bovine immune cells (polymorphonuclear leukocytes or PMNs, lymphocytes, monocytes). 72 milk samples from Italian Brown Swiss cows were collected and classified into four groups based on SCC and DSCC values, determined using a Fossomatic™ 7DC analyser. Immune cells were stimulated with PHA (lymphocytes) or LPS (PMNs and monocytes) to evaluate miRNA expression and nitric oxide production. miR-26a showed a downregulation in susceptible (0.7-fold) ($p < 0.05$), chronic (0.8-fold) ($p < 0.01$) and acute mastitis (0.8-fold) ($p < 0.01$) groups, suggesting its potential utility as an early inflammation biomarker. miR-223 was significantly upregulated only in the acute mastitis group (*vs.* control: 26.5-fold, *vs.* susceptible: 24.4-fold, *vs.* chronic: 25.7-fold) ($p < 0.01$). These expression profiles, together with DSCC values, were integrated into a diagnostic decision model that can discriminate between different mastitis stages. In immune-cell cultures, miR-26 exhibited reduced expression following lipopolysaccharide stimulation in neutrophils (0.4-fold) ($p < 0.01$), whereas miR-223 shows increased expression in lymphocytes activated with phytohemagglutinin (0.2-fold) ($p < 0.05$).

In conclusion, this thesis contributes to a better understanding of the interaction mechanisms between the immune system and the mammary gland, identifying melatonin and c-miRNAs as promising and non-invasive indicators of udder health. Their use in combination with SCC and DSCC could improve the accuracy and timing of mastitis diagnosis and prevention, supporting farming approaches aimed at reducing antibiotic use and improving animal welfare.

List of abbreviations

AANAT = arylalkylamine N-acetyltransferase

ACTH = adrenocorticotropic hormone

aDCT = antibiotic dry cow therapy

AM = acute mastitis group

APC = antigen presenting cell

ASMT = acetylserotonin O-methyltransferase

cDNA = complementary DNA

CM = chronic mastitis group

c-miRNA = circulating microRNA

CMT = California Mastitis Test

CNS = central nervous system

CRH = corticotropin-releasing hormone

Ctrl = control group

DIM = days in milk

DSCC = differential somatic cell count

E. coli = Escherichia coli

EC = electrical conductivity

FBS = foetal bovine serum

FFA = free fatty acids

HBSS = Hank's Balanced Salt Solution

HPA = hypothalamic-pituitary-adrenal axis

IRT = infrared thermography

ISCC = in-line somatic cell count

LPS = lipopolysaccharide

miR-142 = miR-142-5p

miR-146 = miR-146a

miR-223 = miR-223-3p

miR-26 = miR-26a-5p

miRNA = microRNA

mRGC = melanopsin retinal ganglion cell

NO = nitric oxide

PBMC = peripheral blood mononuclear cell

PBS = phosphate buffered saline solution

PHA = phytohemagglutinin

PMN = polymorphonuclear leukocyte

qPCR = real-time quantitative Polymerase Chain Reaction

RISC = RNA-induced silencing complex

S. agalactiae = Streptococcus agalactiae

S. aureus = Staphylococcus aureus

SCC = somatic cell count

SCN = suprachiasmatic nucleus

SU = susceptible group

1. Introduction

The work presented in this chapter has been partially published in Dall'Olio et al. (2026) – Functional Role and Diagnostic Potential of Biomarkers in the Early Detection of Mastitis in Dairy Cows, Animals, 16, 159.

1.1 Bovine mastitis: a persistent challenge in modern dairy herds

In recent years, the bovine intensive farming system use has increased significantly, leading to a deterioration in the health and welfare of dairy cows, mainly due to a higher incidence of metabolic disorders, such as laminitis and ketosis, and inflammatory diseases, in particular mastitis (Raboisson et al., 2016; Zachut et al., 2020). Mastitis is, in fact, the only dairy cattle disease that can directly reduce the commercial value of milk (Ruegg, 2012; Puerto et al., 2021).

The first documented reference to bovine mastitis dates back to 1917, when Breed and Brew (1917) highlighted the public health risks associated with high bacterial concentrations in raw milk. Despite more than a century having passed and considerable progress being made in terms of cow management and knowledge of mastitis, this disease continues to represent a major challenge in dairy production (Ruegg, 2017).

Mastitis represents a major problem for the dairy industry both from an economic and animal welfare point of view. Mastitis can lead to a reduction in milk production and quality (Zecconi et al., 2023), and it is also one of the major reasons for the antibiotics use, contributing to the worsening of antibiotic resistance (Sharun et al., 2021; Zecconi et al., 2023). Milk from mastitic cows shows a higher somatic cell count (SCC), making it unsuitable for consumption and commercialisation. Bovine mastitis is estimated to result in daily milk losses of 1.0 to 2.5 kg per cow during the first two weeks following disease onset, with total losses over the entire lactation period ranging from 110 to 552 kg per cow (Sharun et al., 2021). These production losses are further compounded by additional expenses related to treatment costs and the premature culling of mastitic cows (Romero et al., 2018; Libera et al., 2021). Specifically, approximately 49% of the total financial losses attributable to mastitis are due to reduced production and increased milk discard, while 37% are associated with culling and treatment costs (Sharun et al., 2021). On average, the total cost of mastitis has been estimated at $ 147 per cow per year, mainly caused by milk production losses and culling, which together account for 11% to 18% of the gross margin per cow per year (Jamali et al., 2018; Hogeveen et al., 2019). It is crucial to note that mammary tissue damage, which leads to reduced milk production, has been reported to contribute approximately 70% of the total economic losses associated with mastitis, as damaged tissue can be often unable to return to its full function (Zhao and Lacasse, 2008).

Neave et al. (1966) conducted on field studies to evaluate the impact of implementing a comprehensive hygiene protocol on dairy farms. They demonstrated that the adoption of hygiene practices such as pre-milking teat disinfection, the use of milking gloves, and post-milking teat immersion significantly reduced new intramammary infections. Moreover, even after the implementation of a "partial hygiene" protocol, which, unlike the complete protocol, does not include teat cup disinfection between cows, a 44% reduction was still achieved. Additionally, they recommended antibiotic treatment at dry-off to further reduce the infection risk. These findings formed the basis of modern mastitis control strategies and significantly improved udder health and milk quality on a global scale.

The National Mastitis Council in the United States, based on the Neave et al. (1966) work just described, developed the "5-Point Plan" as a strategic approach for the control and prevention of bovine mastitis. This program includes post-milking teat disinfection, the systematic use of dry cow therapy at the end of each lactation, timely treatment of clinical cases, culling of cows with chronic mastitis, and proper maintenance and sanitisation of milking equipment to ensure adequate vacuum levels at the teat end.

In addition to its direct impact on udder health, mastitis has also been associated with reduced fertility and prolonged calving-pregnancy intervals in cows. These reproductive deficits have been observed even in cases of mild inflammation (Huszenica et al., 2005; Lavon et al., 2016). Consequently, the dairy industry is progressively adopting mastitis prevention strategies focused on rigorous hygiene protocols, including the aforementioned "5-Point Plan", with the aim of minimizing antibiotic use and reducing economic losses related to reduced milk quality and treatment costs. Indeed, as Murphy (1956) highlighted, relying exclusively on antibiotic therapy for mastitis control is like "merely cutting the tops off the weeds and leaving the roots".

1.2 Mastitis in dairy cows: from pathogen invasion to subclinical and clinical outcomes

Given the substantial impact of mastitis on animal health, productivity, and herd management discussed above, a deeper understanding of the pathological processes driving this condition is warranted.

Bovine mastitis, an inflammatory condition affecting the mammary gland, can be triggered by several factors. Bacterial pathogens are the primary etiological agents, although the disease can occasionally be caused by viruses, mycoplasmas, algae, or fungi (Libera et al., 2021; Merin et al., 2024). The pathogenesis of mastitis has been historically described as divided into a three-step progression (Murphy, 1947): initially, occurs microbial invasion of the mammary gland; once bacterial colonization has occurred, the infection phase begins, which finally triggers the characteristic inflammatory response. This framework, originally proposed in the mid-20th century, still underpins the current understanding of mastitis development.

Predominant causative bacterial agents include *Staphylococcus aureus* (*S. aureus*), environmental streptococci (*Streptococcus agalactia, Streptococcus uberis* and *Streptococcus dysgalactie*), coliform bacteria such as *Escherichia coli* (*E. coli*) and *Klebsiella* species, as well as other Gram-negative microorganisms (Carrillo-Casas and Miranda-Morales, 2012; Sharun et al., 2021; Luo et al., 2022; Freu et al., 2023).

Mastitis infections are classified into two categories based on their epidemiological characteristics: contagious mastitis, which occurs when a healthy cow becomes infected through contact with the milker hands or milking equipment contaminated by a previously milked infected animal; environmental mastitis, which is caused by pathogens originating from the farm environment, such as bedding, soil, or faecal matter, and unrelated to the milking process (Sharun et al., 2021). It is also important to highlight that non-infectious factors can significantly influence both the onset and the severity of mastitis. In fact, there are also other variables that can contribute to the development and progression of the disease, such as the animal genetic background, environmental conditions, feed composition, and the inclusion of specific nutritional supplements (Libera et al., 2021; Algharib et al., 2024).

Mastitis can also be classified as clinical or subclinical mastitis, based on a clinical perspective. Clinical mastitis is characterized by visible signs of mammary gland inflammation, including pain, swelling, heat, and redness of the affected quarter, along with visible changes in milk, such as altered colour, abnormal consistency, and clots (Lehtolainen et al., 2004; Antanaitis et al., 2021; Sharun et al., 2021). Systemic symptoms such as fever (body temperature exceeding 39.5°C) and reduced appetite may also occur (Sharun et al., 2021). This mastitis form can be further classified as hyperacute, acute, or subacute based on the severity and progression of these clinical manifestations (Ashraf and Imran, 2020). Clinical mastitis is typically associated with the isolation of coliforms, Gram-negative bacteria, and environmental streptococci (Ji et al., 2022). Variability in clinical severity is closely related to the specific pathogen involved: Gram-negative bacteria, such as *E. coli*, typically elicit a rapid and intense immune response; Gram-positive bacteria are associated with a slower and moderate response; *S. aureus* may trigger a minimal or even undetectable immune reaction (Ashraf and Imran, 2020). Clinical mastitis is particularly problematic due to its recurrent pattern. In fact, cows affected by clinical mastitis during lactation have a higher risk of subsequent episodes during the same period, and a history of clinical mastitis increases susceptibility to new intramammary infections, regardless of the pathogen involved (Jamali et al., 2018).

In contrast, subclinical mastitis, which can affect approximately 25 – 35% of cows within a herd (Owens et al., 2001), is more challenging to diagnose, as it does not present with visible symptoms. This form of mastitis is most associated with non-*aureus* staphylococci and environmental streptococci (Ji et al., 2022). *Staphylococcus chromogenes* has been identified as the predominant etiological agent, although contagious pathogens such as *S. aureus* and *S. agalactiae* have also been isolated (Freu et al., 2023). To date, bacteriological analysis represents the *gold standard* method for the subclinical mastitis diagnosis. However, its practical application is often limited by the high costs and time required for analysis, which are often incompatible with the rapid operational needs of dairy farming. To overcome these issues, indirect methods for the diagnosis of subclinical mastitis have been identified, which are based on alteration in milk composition, typically identified by the SCC or indirect count methods applicable directly in the field (Ashraf and Imran, 2020; Sharun et al., 2021; Antanaitis et al., 2021; Merin et al., 2024), as described in the next section.

1.3 Current strategies and diagnostic challenges in mastitis control

Although several diagnostic methods are currently available for the detection of mastitis, including bacteriological and indirect milk-based approaches, important limitations persist in terms of feasibility, timeliness, and accuracy under field conditions (Dall'Olio et al., 2026).

Over the years, several prevention and treatment of mastitis strategies have been implemented with the aim of enhancing health, welfare, and productivity of dairy cows (Ismail, 2017; Weigel and Shook, 2018; Rainard et al., 2022).

Antibiotic administration represents the most widely adopted strategy for the prevention and treatment of mastitis (Halasa et al., 2007; Saini et al., 2012). However, restrictions on antibiotic use are being implemented in livestock production, due to the worsening of antibiotic resistance and increased attention to animal welfare (Merin et al., 2024). Considering this, it would be of great importance to find new strategies to improve herd management practices for mastitis prevention, and to develop increasingly accurate and early diagnostic tools that allow for timely and targeted interventions. For example, there is a growing trend toward the implementation of selective antibiotic dry cow therapy (aDCT) at the end of lactation, in contrast to the traditional aDCT approach. Selective aDCT targets only cows identified as infected or at risk of infection, avoiding unnecessary treatment of healthy animals (Niemi et al., 2022; Rowe et al., 2023).

In recent decades, the development of targeting common udder pathogens vaccines has been significantly improved, and significant progresses has been made in preventing mastitis through genetic selection for increased resistance to infections (Odegård et al., 2003; Ikonen et al., 2004; Hinrichs et al., 2005; Heringstad et al., 2006; Cha et al., 2016; Collado et al., 2016; Misra et al., 2018; Zhylkaidar et al., 2021; Weigel and Shook, 2018; Rainard et al., 2022). However, both strategies face challenges, primarily due to the high number of pathogens that can cause intramammary infections. Both commercially available vaccines and herd-specific autovaccines, based on inactivated bacterial cells, are routinely used in dairy herd management, but their efficacy often remains limited and, in many cases, insufficient (Ismail, 2017).

Chakraborty et al. (2019) review offers a comprehensive overview of all currently available diagnostic methods for mastitis.

SCC is a widely used and well-established method for detecting mastitis, offering rapid results at relatively low cost (Džermeikaitė et al., 2023). This direct counting technique involves microscopic analysis of milk samples stained with methylene blue (Ashraf and Imran, 2020). Currently, a threshold of 2.0×10^5 cells/mL is commonly used to indicate subclinical mastitis, while the 4.0×10^5 cells/mL threshold il for clinical mastitis diagnosis (Piccinini et al., 2005; Antanaitis et al., 2021; Dall'Olio et al., 2025). An evolution of the SCC method emerged from adapting the technique to the needs of large-scale farms, where quickly and easily monitoring every single animal was not feasible. This led to the development of in-line somatic cell count (ISCC) monitoring, an automated procedure based on the electrical conductivity (EC), ISCC significantly reduces the occurrence of false positive results compared to using either method alone (Kamphuis et al., 2008).

Indirect diagnostic methods are also available to detect SCC, including the California Mastitis Test (CMT), firs described in 1957 (Schalm and Noorlander, 1957) and still widely used today for its simplicity and rapid field applications. According to the method developed by Schalm and Noorlander (1957), the CMT is performed by collecting 2 mL of milk from each udder quarter and mixing it with a reagent containing 3% sodium lauryl sulphate and 1:10,000 bromocresol purple. The formation of gel-like thickenings in the mixture is proportional to the somatic cell concentration, but the test relies on a visual and subjective interpretation of results (Ruegg, 2017). Despite their widespread use, both SCC and CMT present significant limitations. Specifically, CMT, as an indirect test, can easily yield false positive and false negative results (Ashraf and Imran, 2020) and is heavily influenced by the operator subjectivity, as operators only perform a visual assessment of the milk sample.

Regarding SCC, although considered more reliable than CMT, it cannot be used directly in the farm environment, but require laboratory assistance, in contrast with CMT, which is a on field test, and can also produce false-positive or negative results (Valldecabres et al., 2023). In fact, SCC can remain below the diagnostic threshold during the early stages of clinical or subclinical mastitis, potentially delaying intervention (Zecconi et al., 2019). Furthermore, it should be considered that somatic cells include not only immune but also epithelial cells, mainly due to the physiological sloughing of mammary tissues, and their concentration can be influenced by factors unrelated to infection, such as lactation stage, herd management practices, and pathological conditions such as inflammation (Alhussien and Dang, 2018; Mondini et al., 2025). To overcome the epithelial cells influence on the quantitative data provided by SCC, a new, more detailed marker of udder inflammation has recently been identified: the differential somatic cell count (DSCC), which can provide qualitative data by measuring the percentages of polymorphonuclear leukocytes (PMNs, or neutrophils) and lymphocytes out of the total amount of somatic cells in milk (Halasa and Kirkeby, 2020). The DSCC threshold that allows to distinguish between healthy and mastitic cows (which have and activated immune system) is 68.5% (Zecconi et al., 2019, 2023). Lymphocytes modulate immune responses, while PMNs play a key role in eliminating pathogens through phagocytic and enzymatic activity (Rosales, 2018; Halasa and Kirkeby, 2020). Monocytes are also active components of the immune system during infections and inflammation, which once in situ differentiate into proinflammatory macrophages (M1), which participate in cytokines and chemokines release, or anti-inflammatory macrophages (M2), which eliminate pathogens through phagocytosis (Arora et al., 2018; Halasa and Kirkeby, 2020; Ceciliani et al., 2021). In healthy cows, lymphocytes are the predominant component (75%) of somatic cells, followed by monocytes/macrophages (18%), PMNs (5%), and epithelial cells (2%) (Dall'Olio et al., 2025). Mastitis, both subclinical and clinical, is characterized by a marked increase in PMNs (up to 55 – 65%) and monocytes/macrophages (28 – 36%), and a significant reduction in lymphocytes (3 – 6%) (Sarikaya et al., 2006; Halasa and Kirkeby, 2020; Zecconi et al., 2023). The combined use of DSCC with SCC parameters improves the diagnosis accuracy of the latter (Zecconi et al., 2023).

Infrared thermography (IRT) is a more recent diagnostic tool that offers a non-invasive method to recognise udder inflammation, also allowing for the distinction between clinical and subclinical mastitis (Chakraborty et al., 2019). It works by detecting temperature differences on the skin of the udder or teat. Dairy cows with acute inflammation show higher temperature than healthy cows, and, in particular, those with clinical mastitis tend to show the highest surface temperature, while those with subclinical mastitis show altered but lower temperature in relation to cows with the clinical form of the disease. However, although IRT is more sensitive the CMT, it is considerably less specific.

Overall, there is a growing need to focus on optimizing herd management strategies for mastitis prevention, developing innovative and more sensitive diagnostic methods that can enable early diagnosis and support timely and targeted interventions in dairy cattle.

1.4 Melatonin and the udder health: synthesis, light-driven secretion and functions

Regarding the udder health management in dairy cows on intensive farms, there is a growing interest in new and improved approaches to prevent infections and resulting inflammation. Recently, this interest has increasingly focused on the immunomodulatory role of melatonin and its circadian regulation, with a view to improving udder health, resulting in a possible reduction of risk of mastitis.

Melatonin ($C_{13}H_{16}N_2O_2$) is an indolamine hormone derived from tryptophan and synthesized by the pineal gland (Tan et al., 2014). Its biosynthesis begins with the conversion of tryptophan to serotonin, which is then acetylated by arylalkylamine N-acetyltransferase (AANAT) and finally methylated by acetylserotonin O-methyltransferase (ASMT) to form melatonin (Xie et al., 2022). AANAT enzyme activity is low during the day and increases in darkness, promoting melatonin production (Hwang et al., 2022). Melatonin synthesis is controlled by a multisynaptic pathway involving nocturnal norepinephrine release from sympathetic fibers, which activates AANAT via β1-adrenergic receptors (increasing cAMP) and α1-adrenergic receptors (modulating intracellular calcium), though calcium role varies among species (Andrani et al., 2024).

Melatonin secretion follows a circadian rhythm but declines with age due to reduced pineal innervation, receptor loss, and calcification (Andrani et al., 2024). Unlike other hormones, melatonin is not stored in the pineal gland, but is synthesized exclusively during night (dark) hours to regulate circadian rhythms and physiological balance (Masters et al., 2014; Amaral and Cipolla-Neto, 2018). After its synthesis, melatonin is secreted into the bloodstream, where it binds to albumin and is transported to the central nervous system (CNS) and peripheral tissues, where it exerts various effects before being catabolized through hydroxylation and oxidation (Amaral and Cipolla-Neto, 2018; Mannino et al., 2021). Its release is controlled by the suprachiasmatic nucleus (SCN), which is regulated by light received by the retina (Hastings et al., 2008; Von Gall, 2022): melanopsin retinal ganglion cells (mRGCs), sensitive to blue light (460 – 480 nm), inhibit melatonin synthesis (Figure 1) during diurnal hours in response to blue light stimulation of the retina (Andrani et al., 2024). Daytime blue light is essential for alertness, but at night can disrupt circadian rhythms and negatively affect sleep quality (Wahl et al., 2019).

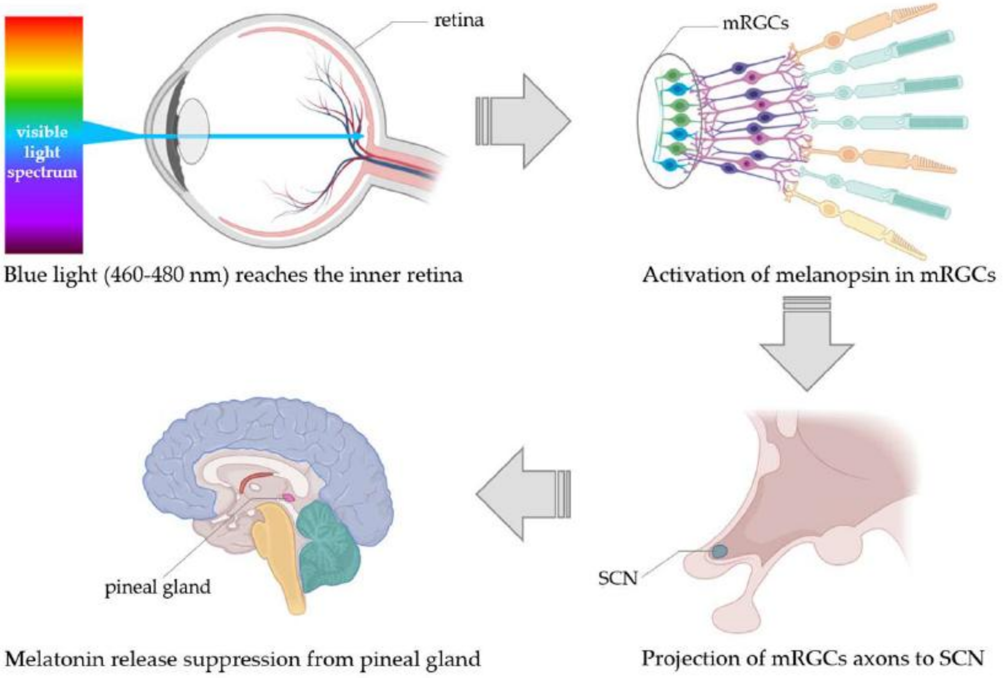


Figure 1 Schematic representation of the blu-light-dependent suppression of melatonin release from our review (Andrani et al., 2024). Blue light within the 460 – 480 nm range reaches the inner retina and activates melanopsin, expressed in intrinsically photosensitive melanopsin-containing retinal ganglion cells (mRGCs). Upon activation, mRGCs transmit signals through their axonal projections to the suprachiasmatic nucleus (SCN), the main circadian pacemaker (Hastings et al., 2018). SCN stimulation subsequently inhibits pineal gland activity, resulting in the suppression of nocturnal melatonin secretion.

Melatonin, as an amphiphilic molecule, can exert its actions through both receptor-mediated and receptor-independent mechanisms. Its main role is the regulation of the sleep-wake cycle, which it achieves by suppressing wake-promoting signals in the SCN in mammals (Amaral and Cipolla-Neto, 2018). Its receptor-mediated actions involve various intracellular targets, including enzymes, transporters, cytoskeletal elements, and mitochondria (Andrani et al., 2024). In addition, as receptor-independent actions, melatonin can directly scavenge free radicals, exert antioxidant effects by inhibiting pro-oxidative enzymes, promote glutathione synthesis, and supports mitochondrial function. Melatonin is also able to regulate the functioning of the hypothalamic-pituitary-adrenal (HPA) axis, which is essential for the stress response: during dark hours, melatonin inhibits the secretion of corticotropin-releasing hormone (CRH) at the hypothalamic level, thus determining a decrease in adrenocorticotropic hormone (ACTH) concentrations and, consequently, cortisol levels (Ciani et al., 2021). In fact, cortisol release also follows a circadian rhythm which, in contrast with melatonin, peaks in production approximately 30 minutes after waking up (Focke and Iremonger, 2020). Melatonin also contributes to reduce body temperature, blood pressure, and alertness via hypothalamic pathways (Reiter et al., 2009). Additionally, it also has anti-inflammatory, neuroprotective and immunomodulatory effects, probably due to its action in reducing oxidative stress (Andrani et al., 2024). Melatonin modulates the immune system by stimulating immune cells and regulating cytokine production, supporting immune function and controlling excessive responses in autoimmune diseases; moreover, melatonin inhibits pro-inflammatory pathways, potentially alleviating symptoms of inflammatory conditions (Carrillo-Vico et al., 2013; Andrani et al., 2024).

1.5 Biogenesis, functional role and diagnostic potential of microRNAs in inflammation

In recent years, research has also increasingly focused on the study of microRNAs (miRNAs), some of which appear to play a role in regulating inflammatory processes. The discovery of the first miRNA in *Caenorhabditis elegans* in the 1990s (Wightman et al., 1993; Lee et al., 1993) and the characterization of these molecules earned researchers Victor Ambros and Gary Ruvkun the 2024 Nobel Prize in Medicine.

MiRNAs are short, non-coding RNA molecules approximately 20–22 nucleotides long that regulate post-transcriptional gene expression and are involved in several biological processes (Miretti et al., 2020). MiRNAs are produced by various cell types, from which they are secreted into the extracellular environment and into biological fluids, such as milk, where they regulate cellular functions. miRNAs biosynthesis (illustrated in Figure 2) begins with the cleavage of the primary transcript (pri-miRNA) in the nucleus by the Microprocessor complex, which includes the RNase Drosha and the protein DGCR8, which functions to transport Drosha to the pri-miRNA (Zong et al., 2022). Following cleavage, a precursor miRNA (pre-miRNA) is generated, which has the characteristic hairpin structure. This precursor is transported by the transporter protein Exportin-5 into the cytoplasm, where it is further modified by the endoribonuclease Dicer to produce the mature miRNA (Winter et al., 2009; Zong et al., 2022). The mature miRNA becomes part of the RNA-induced silencing complex (RISC), where it exerts its regulatory function by binding to target mRNA molecules, resulting in their degradation or gene silencing (Lawless et al., 2014; Frédérick and Simard, 2022).

The main miRNA target mRNAs synthesize regulatory proteins, such as signalling molecules, transcription factors, and cell death regulators. Indeed, miRNAs fine-tune protein expression, regulating inflammatory responses. Following the characterization of their role in tumour cells, they are currently used as biomarkers in human medicine, thanks in part to their non-invasive nature, as they can be detected in all body fluids, such as plasma and urine (O'Connell et al., 2012). Furthermore, their synthesis is closely influenced by inflammatory stimuli.

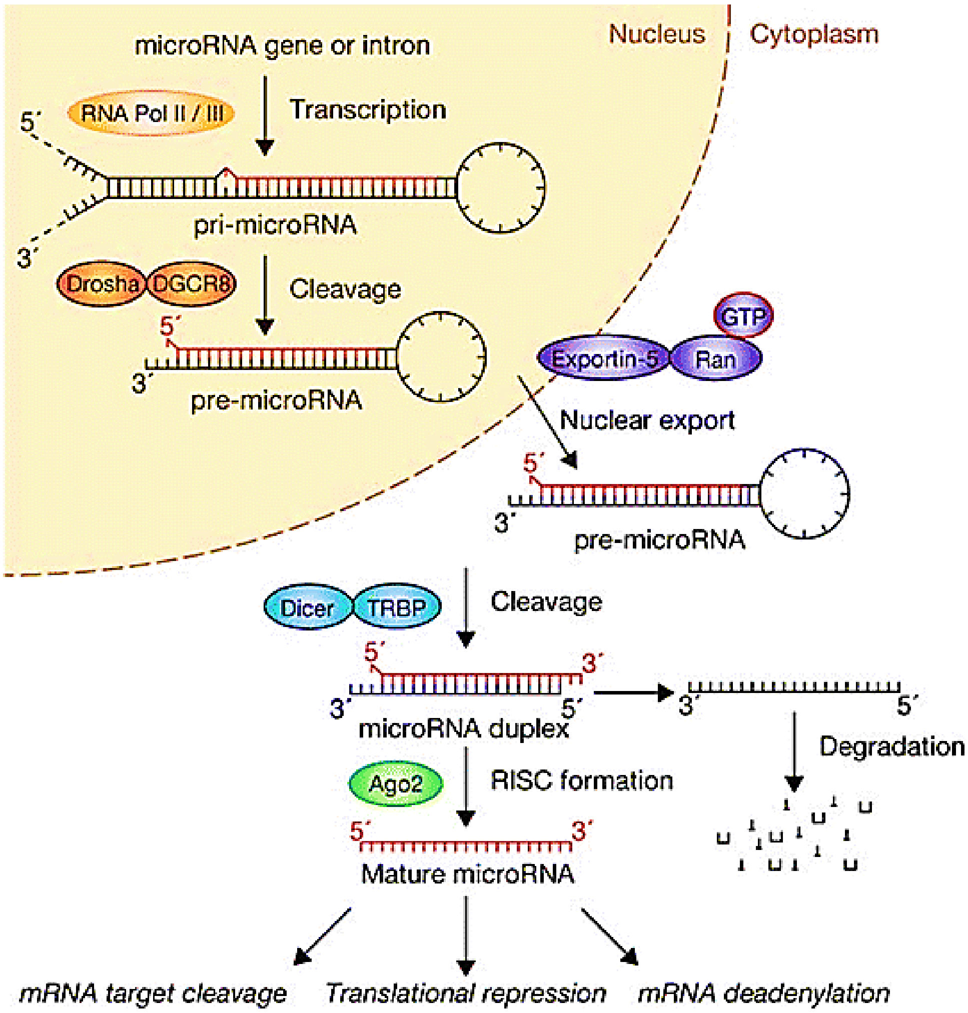


Figure 2 Overview of the microRNA (miRNA) biogenesis pathway, illustrating the sequential processing steps from the nucleus to the cytoplasm from Winter et al. (2009). Primary miRNA transcripts (pri-miRNAs), produced by RNA polymerase II or III, undergo initial cleavage by the Drosha-DGCR8 microprocessor complex to generate precursor miRNAs (pre-miRNAs). These hairpin structures are subsequently exported to the cytoplasm through the Exportin-5/Ran-GTP transport system. In the cytoplasm, Dicer in association with TRBP cleaves the pre-miRNA to form a miRNA duplex. One strand of the duplex is selectively incorporated into the Argonaute-containing RNA-induced silencing complex (RISC), yielding the mature, functional miRNA. The mature miRNA then mediates post-transcriptional gene regulation through mRNA target cleavage, translational repression, or mRNA deadenylation.

Biomarkers are biological indicators that can be quantified and can generate predictions about clinically significant outcomes, allowing for faster and less invasive assessment (Aronson and Ferner, 2017). Based on their intrinsic characteristics, biomarkers can be useful for screening, diagnosis, monitoring, and assessing therapeutic response or prognosis of diseases. However, to be effective, they must have both high specificity and sensitivity, demonstrating rapid and specific changes in their concentrations (Lee et al., 2006; Aronson and Ferner, 2017).

A typical characteristic of circulating miRNAs (c-miRNAs) found in milk is their high stability: they are resistant to degradation by RNAses, the acidic conditions of milk, and possible freeze-thaw cycles (Lai et al., 2017). Furthermore, their secretion into the extracellular environment and their changes in expression following the development of pathological conditions represent a further aspect that identifies their potential as biomarkers for animal health and welfare (Miretti et al., 2020; Zhang et al., 2021).

In the context of inflammation, and specifically mastitis in cows, specific miRNAs have been identified and correlated with the inflammatory process, highlighting them as potential biomarkers for the diagnosis and monitoring of bovine mastitis. Increased levels of miR-21, miR-146a, miR-155, miR-222, and miR-383, miRNAs involved in the regulation of immune and inflammatory pathways, have been detected in milk samples from cows with mastitis. Their expression has been shown to be positively correlated with CMT score (Sheedy et al., 2010; Wang et al., 2016; Lai et al., 2017; Song et al., 2017; Jadhav et al., 2024). Studies by Lai et al. (2017, 2021) therefore appear to support the hypothesis that the expression of inflammation-related miRNAs also responds to stimuli resulting from mastitis and suggest that mastitis also appears to influence the expression of c-miRNAs in milk. Srikok et al. (2020) also observed variations in the expression of some miRNAs in the milk of cows with mastitis, specifically they highlighted an upregulation of miR-29b-2 and miR-184 and a downregulation of miR-146a, miR-148a and miR-155. The researchers in this study indicated miR-29b-2 as a promising early diagnostic biomarker, as it showed good characteristics of specificity (81%), sensitivity (96%) and accuracy (89%).

Luoreng et al. (2018) observed variations in the expression of specific miRNAs in milk following the experimental induction of mastitis by *S. aureus* or *E. coli*. Specifically, they detected an overexpression of miR-144 and miR-451 in cows infected with *S. aureus*, and a downregulation in samples from cows with *E. coli*-induced mastitis, suggesting that miRNAs as biomarkers may also allow the distinction between etiological agents causing mastitis. miR-7863 was overexpressed in samples from both groups, indicating its more general character as a potential generic biomarker of mastitis, likely involved in the regulation of interleukin pathways (Luoreng et al., 2018).

In a subsequent study, Luoreng et al. (2021) determined the blood miRNA profile of cows with *S. aureus*-induced mastitis. This profiling revealed that miR-1301 and miR-2284r exhibited significant alterations in their expression concomitant with infection, indicating their potential as biomarkers for *S. aureus*-induced mastitis.

Finally, four c-miRNAs, specifically miR-26a, miR-142-5p, miR-146a, and miR-223, were identified as overexpressed in milk samples from cows with subclinical mastitis and were positively correlated with the CMT score and the presence of pro-inflammatory cytokines, such as IL-1β and TNF-α (Tzelos et al., 2022). It was also observed that the lactation phase appears to affect the expression of these four c-miRNAs, with a tendency for higher amounts in early lactation. Through ROC curve analysis, it was then determined that miR-142-5p, miR-146a, and miR-223 exhibited a high degree of diagnostic accuracy, supporting their potential role as biomarkers for mastitis.

Therefore, given all their characteristics reported so far, c-microRNAs can be considered promising diagnostic biomarkers, thanks primarily to the fact that their expression is finely regulated both temporally and spatially (levels change rapidly and in specific regions, such as in milk) and that they are secreted into all body fluids (easily quantified noninvasively), where they are particularly stable.

2. Aim of the thesis

This thesis explored two pathways involved in the regulation of bovine udder health: the role of melatonin and the expression of selected circulating microRNAs (c-miRNAs) in milk.

Experiment 1 primarily assessed variations in melatonin concentration in milk samples collected in winter, comparing those from daytime and nighttime milkings. Moreover, SCC and DSCC parameters were also assessed to evaluate and further explore the possible mechanisms underlying melatonin immunomodulatory role.

Experiment 2 instead evaluated the expression of four c-miRNAs (miR-26a-5p, miR-142-5p, miR-146a, and miR-223-3p) already associated with the inflammatory process of the mammary gland, through in vivo analysis in milk and in vitro analysis in the supernatant of inflammatorily stimulated immune cells (lymphocytes, PMN and monocytes) monocultures. The aim of the experiment 2 was to evaluate the potential of these c-miRNAs as potential early biomarkers of mastitis.

Overall, these two studies aimed to deepen the understanding of mammary gland immune system regulation and identify novel, non-invasive molecular tools to improve the early diagnosis and management of mastitis in dairy cows.

3. Experiment 1 – Less light, healthier milk: melatonin, a natural shield against mastitis?

The content presented in this chapter is based on material included in an international published review, in which I am a co-author, and in a research article in press: Andrani et al. – Bioactive peptides in dairy milk: highlighting the role of melatonin, Biomolecules 2024, 14, 934 (co-authored); and Dall'Olio et al. – Nighttime or daytime? How milk melatonin levels relate to differential and somatic cell count in dairy cow milk, The Thai Journal of Veterinary Medicine (in press).

3.1 Introduction

In dairy cows, as in other mammals, melatonin is secreted into milk following a circadian rhythm, with higher production at night and higher concentrations in early morning milkings (Andrani et al., 2024). However, available studies on melatonin content in bovine milk are limited, and reported values (Table 1) vary because of numerous factors such as season, latitude, breed, milking frequency, and nutritional status. Nighttime melatonin levels are typically higher, especially in winter compared to summer (Asher et al., 2015; Boztepe et al., 2022), and breed differences also play a role: for example, Holstein and Jersey cows show day-night variations, with melatonin concentrations almost doubled in the nighttime milk of Holstein cows (Boztepe et al., 2022). Artificial lighting in barns can also significantly affect melatonin production. Extended dark periods and low-intensity nighttime lighting increase melatonin in cows milk (Asher et al., 2015; Andrani et al., 2024). Furthermore, exposure to blue LED light suppresses melatonin more than yellow light, due to the sensitivity of melanopsin to blue wavelengths (Elsabagh et al., 2020), as illustrated in Figure 1. Melatonin levels can be increased by extending darkness hours, adjusting light wavelengths, and limiting nighttime light intensity to less than 10 lux (Asher et al., 2015; Romanini et al., 2019).

Table 1 Summary of published data reporting melatonin concentrations in milk collected during daytime and nighttime milking across different studies and lighting regimens. The table compares melatonin levels measured in cows of various Holstein herds under distinct photoperiod conditions, including natural seasonal light-dark cycles, controlled artificial light-dark schedules, and periods of continuous darkness. Reported values highlight the consistent increase in melatonin concentration in nighttime milk relative to daytime milk, with variations attributable to experimental design, melatonin quantification system, environmental illumination, and management practices. DN= dark night; IN = illuminated night.

Cows	**Melatonin (pg/mL)**		**Lighting (h)**		**References**
Breed	**Day Milk**	**Night Milk**	**Light**	**Dark**	
30 Holstein	6.9 ± 3.1	14.9 ± 7.7	13.30 summer 10.30 winter	2h light on, 5h light off, 5h light on	Romanini et al., 2019
28 Holstein	5.4 ± 0.3 (DN) 3.3 ± 0.2 (IN)	30.7 ± 1.8 (DN) 17.8 ± 0.3 (IN)	10.40 winter	13.60 winter	Asher et al., 2015
10 Holstein	4.0	39.4	-	-	Milagres et al., 2014
10 Holstein	90.2	120.1	13 summer	11 summer	Teng et al., 2021
40 Holstein	103.7 ± 6.6	163.1 ± 8.9	-	Darkness for a week	Sahin et al., 2021

As previously discussed in paragraph 1.4, melatonin synthesis is strongly influenced by photoperiodism, which refers to the effects of variation in the light-dark ratio on the physiological responses of organisms. In particular, higher melatonin concentrations have been observed in milk collected from nighttime milkings during the winter season, when the duration of daylight il significantly reduced compared to the summer months (Asher et al., 2015; Teng et al., 2021). This regulation of melatonin secretion, which is therefore both seasonal and circadian, is reflected in milk composition and has been the focus of several studies investigating its potential health implications for dairy cows. An increase in the natural melatonin content in milk has been negatively associated with SCC and, therefore, with the risk of developing mastitis (Asher et al., 2015). Additionally, treatments with this hormone, through subcutaneous injections or rumen bypass feeding, also seem to induce a significant reduction in SCC and lower incidence of mastitis (Yang et al., 2017; Yao et al., 2020; Wu et al., 2021).

The conventional approach to reducing SCC relies on the use of antibiotics to manage mammary gland infections. However, this strategy present significant challenges, as it contributes to the rise of antibiotic resistance in livestock production systems and leads to the presence of antibiotic residues in milk, compromising its safety for human consumption and commercial value, as previously mentioned. Consequently, there is growing interest in exploring alternative therapeutic options for mastitis that are non-toxic, have antibacterial properties and can support immune function. Among these, melatonin has been identified as a promising candidate, both by naturally increasing its concentration through photoperiod and artificial lighting management and through exogenous supplementation (Asher et al., 2015; Romanini et al., 2019; Yao et al., 2020; Wu et al., 2021). The positive effects of melatonin could be linked to its immunomodulatory capacity and its role in stimulating antioxidant enzymatic activity (Asher et al., 2015; Yang et al., 2017).

The aim of the study was to evaluate variations in melatonin concentration in milk samples collected at 12-hours intervals (4:00 am vs 4:00 pm) during the winter period in dairy cows on a commercial farm in the Parmigiano Reggiano Consortium. Furthermore, the research also sought to evaluate the relationship between melatonin levels and SCC and DSCC parameters in the same milk samples, to explore and further understand the mechanisms by which melatonin regulates the immune system in the mammary gland.

The study therefore sought to clarify whether the immunomodulatory effect of melatonin on SCC observed in literature could be attributable to a composition remodulation of somatic cells, particularly their immune component, to identify potential new strategies for enhancing mammary gland health naturally and independently of antibiotic treatment.

3.2 Materials and methods

3.2.1 Ethical statements

Experiment 1 was conducted on dairy cows housed at a commercial farm in northern Italy. Milk samples were collected during regular milking sessions performed by ARAER (Regional Breeders Association of Emilia-Romagna) as part of their routine functional monitoring. Sample handling and analysis were carried out according to the standard procedures established for milk recording and testing in dairy cattle.

3.2.2 Animals profile and housing management

The research involved 40 Holstein Friesian cows, with average days in milk (DIM) 174 ± 140, parity of 2.43 ± 1.50, and a mean daily milk production of 26.09 ± 9.21 kg. The animals were housed in pen barns and milked twice a day using milking parlors. Sampling took place in February 2024, during environmental conditions with an average temperature of 11°C (ranging from 9°C to 13°C), 95% relative humidity, and precipitation. The natural light-dark cycle consisted of 11 hours of daylight and 13 hours of darkness. During the nighttime milking, artificial lighting in the barn was switched on, while daytime milking was performed under natural daylight conditions. Feeding protocols followed the guidelines established by the Parmigiano Reggiano

Consortium (Consorzio del Parmigiano Reggiano, 2018), ensuring alignment with the nutritional standards required for Parmigiano Reggiano cheese production.

3.2.3 Milk sampling, analysis, and SCC and DSCC quantification

Milk samples were collected from each experimental cow twice daily: Night milk samples group at 4:00 am and Day milk sample group at 4:00 pm, resulting in a total of 80 samples. For each milking session, one liter of milk per cow was obtained and promptly stored. Milk samples were immediately analysed for composition, including protein, fat, lactose, urea, and casein content, using the MilkoScan FT3 analyser (Foss Electric A/S, Hillerød, Denmark). SCC and DSCC were measured with the Fossomatic 7DC analyser (Foss Electric A/S, Hillerød, Denmark). Subsequently, aliquots of milk were transferred into 15 mL Falcon tubes and frozen at -20°C for later melatonin quantification.

3.2.4 Quantification of melatonin in milk samples

Melatonin levels were quantified using the Melatonin ELISA kit (REF54021; Tecan, Hamburg, Germany), following the manufacturer protocol, as summarized below. Milk samples underwent centrifugation to remove the fat layer: 15 mL aliquots were spun at 5000 × g for 10 minutes, repeated three times, with lipid layers discarded after each spin. Skimming milk is necessary because the ELISA kit requires melatonin extraction on a column and the lipid content would clog the extraction column filter. The resulting skimmed milk was then assayed for melatonin concentration using the ELISA kit, which covers a standard range from 3.0 to 300 pg/mL and demonstrated a standard curve with an efficiency (R^2) of 0.99. According to the manufacturer technical documentation, ELISA assay performance was evaluated by spike-and-recovery analysis, with a reported mean recovery of 102.4% and a range of 83 - 125%.

For sample preparation, standards, controls and undiluted milk samples (each 500 µL, tested in triplicates) were applied to methanol-conditioned columns and centrifuged at 120 × g for 5 minutes. After washing twice with 10% methanol, melatonin was eluted with 1 mL methanol, dried under nitrogen, and reconstituted in 150 µL of bi-distilled water.

Next, 50 µL of each prepared sample, standard, and control were placed into microplate wells together with 50 µL of Melatonin Biotin and Melatonin Antiserum. The plate was incubated for 16 hours at 4°C, then washed and incubated with an Enzyme Conjugate for 2 hours at room temperature. After development with Substrate Solution for 40 minutes, the reaction was stopped, and absorbance was measured at 405 nm using a VICTOR® Nivo™ Multimode Microplate Reader (PerkinElmer, Waltham, MA, USA). Melatonin concentrations were calculated from the standard curve.

3.2.5 Statistical analysis

The sample size for the two groups of milk samples (Night and Day groups) was estimated using the "pwr" package (version 1.3-0) within the R environment (version 4.3.3) and RStudio (release 2023.06.1). The calculation was performed assuming a significant level of 0.05, a statistical power of 80%, and a medium, expected effect size, defined as 0.5.

The comparison between Night and Day milk samples for each parameter (milk composition, melatonin, SCC and DSCC) was conducted using a paired two-tailed t-test. Differences in SCC and DSCC among groups were further evaluated through a one-way ANOVA with Tukey's post hoc test to account for multiple comparisons. The normal distribution of the data was verified using the Shapiro-Wilk test. All statistical analyses were carried out with IBM SPSS Statistics software (version 29.0.1.0, IBM Corp., Armonk, NY, USA), considering results statistically significant at p-value < 0.05.

3.3 Results

3.3.1 Stability of milk composition across the circadian cycle

The milk composition of Day and Night groups samples was analysed by comparing the mean percentages of protein, fat, free fatty acids (FFA), lactose, casein and mean values of urea content. The analysis with the paired two-tailed t-test highlighted an effect of milking time only on milk fat content. In fact, the fat content in the Day group, with mean values of 4.73 ± 0.83%, was significantly higher ($p < 0.0001$) than in the Night group, which showed mean percentage of 3.72 ± 0.68%, as shown in Figure 3. In relation to the lipid content, mean FFA values did not differ ($p = 0.26$) between the groups, being 0.45 ± 0.11% during the day and 0.41 ± 1.03% at night.

Finally, milk samples from both groups showed no significant differences in mean values for: protein content (3.31 ± 0.07% Day group, 3.28 ± 0.06% Night group; $p = 0.08$), lactose (4.59 ± 0.05% Day group, 4.56 ± 0.05% Night group; $p = 0.22$), casein (2.62 ± 0.07% Day group, 2.59 ± 0.07% Night group; $p = 0.12$), and urea (36.06 ± 1.05 mg/dL Day group, 36.73 ± 0.85 mg/dL Night group; $p = 0.16$).

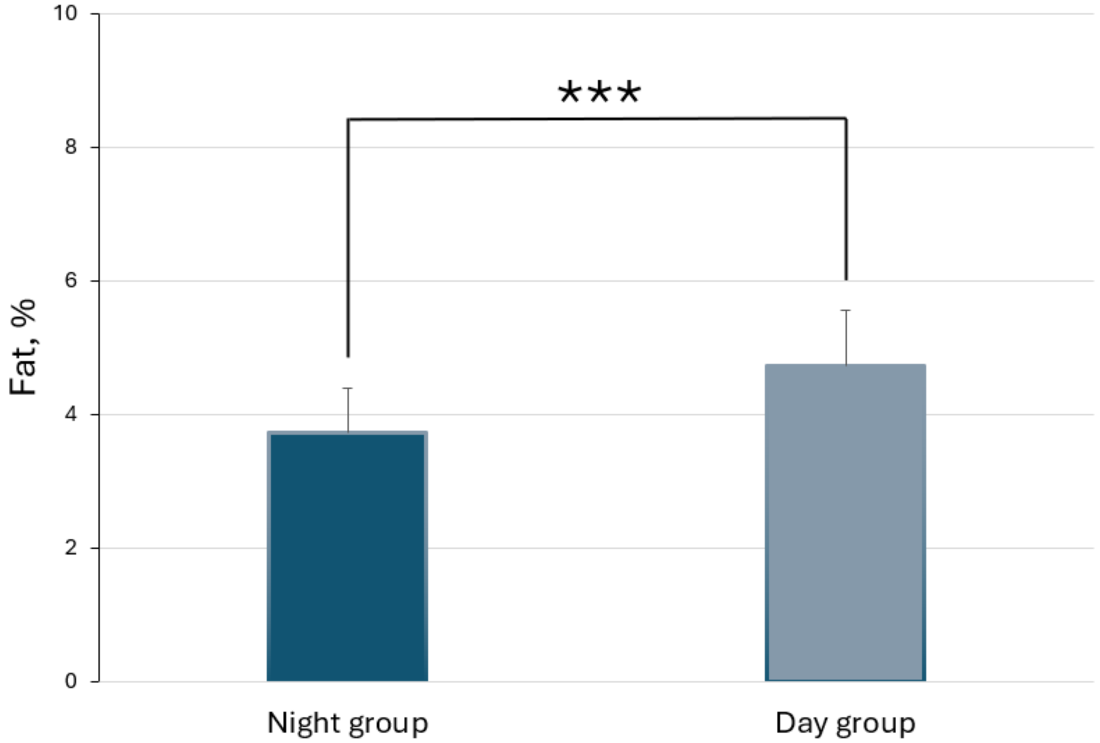


Figure 3 Mean ± SD values of milk fat percentage in samples collected during nocturnal milking (4:00 am) and diurnal milking (4:00 pm). Fat content was markedly higher in the Day-milk group compared with the Night-milk group. *** indicates a significant difference with *p*-value < 0.0001.

3.3.2 Melatonin and somatic cell parameters quantification in milk

Analysis of melatonin and SCC levels in milk revealed an effect of milking time on these two parameters. Melatonin concentrations showed significant variation ($p < 0.001$) between groups, with mean values more than doubled in the Night group (15.63 ± 1.9 pg/mL) compared to the Day group (6.80 ± 0.75 pg/mL), as shown in figure 4A. One-way ANOVA and post hoc tests indicated a reduction in SCC mean values in the Night group compared to the Day group ($p < 0.001$), while DSCC values showed no differences ($p = 0.78$). specifically, nighttime milk samples showed a mean SCC of

129.70 ± 25.01 $\times 10^3$ cells/mL, while daytime milk samples achieved higher values, with a mean of 196.39 ± 33.80 $\times 10^3$ cells/mL, as shown in Figure 4B.

DSCC did not differ between groups, with the percentage of PMN + lymphocytes being 66.54% in the Night group and 63.36% in the Dat group. Correspondingly, the percentage of macrophages + epithelial cells was 33.46% at night and 36.64% in daytime milk. Figure 5 illustrates the proportion of immune cells subpopulations (DSCC) in relation to total amount of somatic cells in milk samples belonging to Day and Night groups.

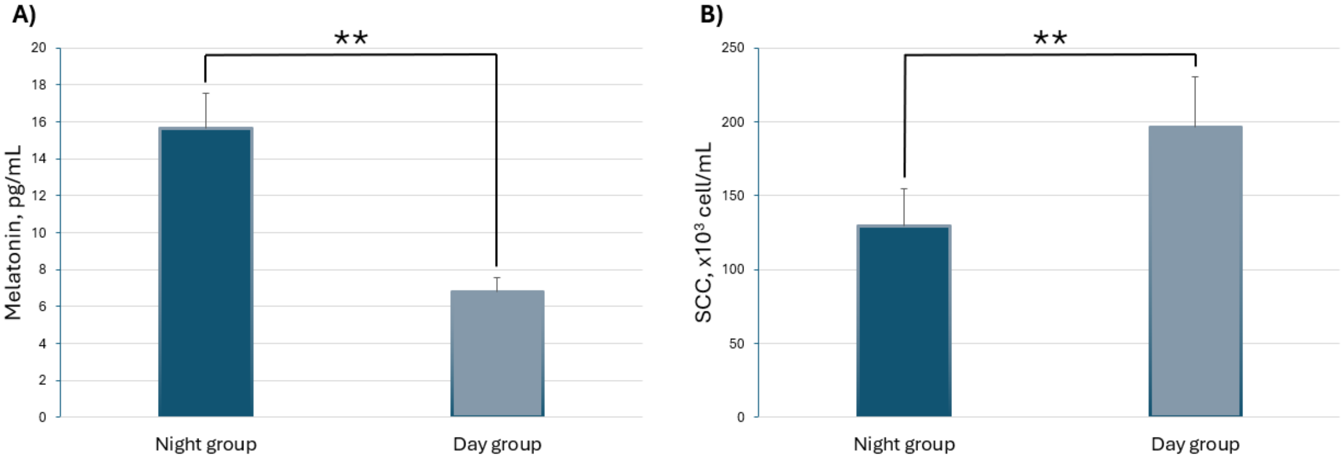


Figure 4 Mean ± SEM values of A) melatonin concentration and B) somatic cell count (SCC) in milk samples collected during nocturnal milking (4:00 am) and diurnal milking (4:00 pm). Night milk samples exhibited significantly higher melatonin concentrations and showed a significant reduction of SCC values. ** indicates a significant difference with p-value < 0.001.

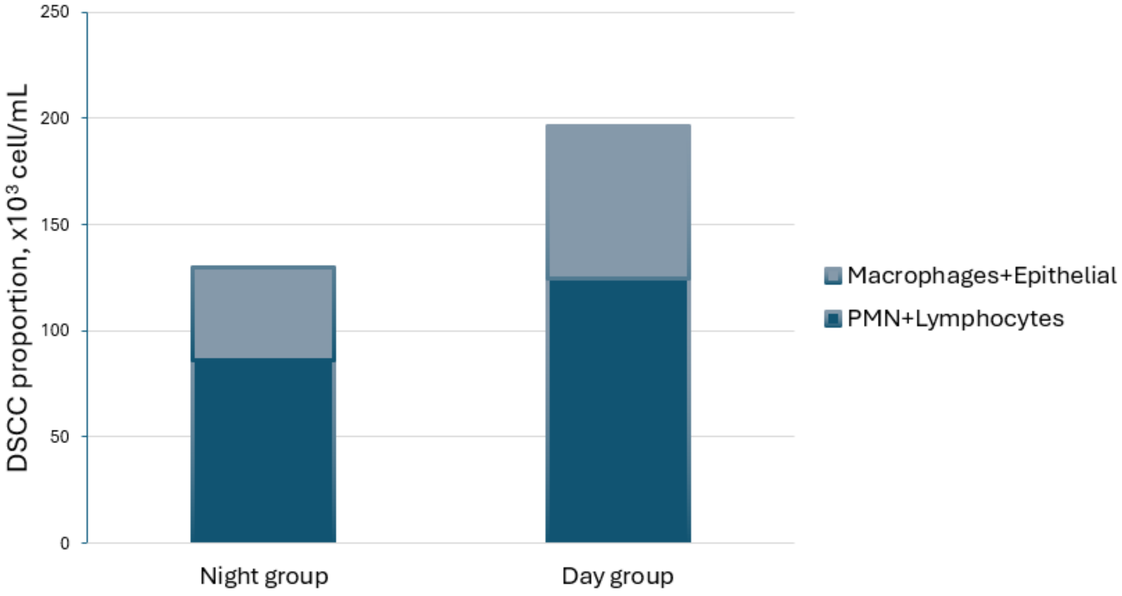


Figure 5 Distribution of immune cell subpopulation in milk collected from cows milked at night (4:00 am) or during the day (4:00 pm), expressed as a proportion of total somatic cells. Differential somatic cell count (DSCC) values represent the combined percentage of polymorphonuclear leukocytes (PMNs) and lymphocytes, while the remaining fraction includes macrophages and epithelial cells. No significant differences were observed between night- and day-milked groups.

3.4 Discussion

In the present research, conducted under a natural photoperiod of approximately 11 hours of light and 13 hours of darkness during the winter season, we observed significantly increased melatonin concentrations in milk samples belonging to Night group (15.63 ± 1.9 pg/mL) compared to those of Day group (6.80 ± 0.75 pg/mL).

This trend is consistent with those reported in the literature (Milagres et al., 2014; Asher et al., 2015; Sahin et al., 2021; Teng et al., 2021) (see Table 1), which demonstrate an effect of milking timing on melatonin content of milk. Furthermore, Romanini et al. (2019), in addition to reporting mean melatonin concentrations in nighttime and daytime milk (14.9 ± 7.7 pg/mL and 6.9 ± 3.1 pg/mL, respectively) similar to the present study, also highlighted a seasonal effect. Milk collected during winter showed significantly higher melatonin levels (13.9 ± 8.4 pg/mL) than samples collected during the summer season (7.9 ± 3.4 pg/mL), indicating a 74.7% increase during winter.

More recent studies have instead documented higher melatonin concentrations, with values reaching 163.1 ± 8.9 pg/mL in nighttime milk and 103.7 ± 6.6 pg/mL during the day (Sahin et al., 2021; Teng et al., 2021). These discrepancies compared to our and previous studies (Milagres et al., 2014; Asher et al., 2015; Romanini et al., 2019) may likely arise from methodological differences in melatonin quantification and animal management: for example, Sahin et al. (2021) kept experimental cows, all in their first lactation, in complete darkness for a whole week before sample collection.

This study (experiment 1) aimed to evaluate potential variations in melatonin levels on a commercial dairy farm without making changes to cow management routine, including lighting management. Milk samples were collected from 40 dairy cattle milked under artificial lighting during the nighttime milking. Despite this, a significant increase in melatonin levels was observed in the Night group. These results suggest that improved management of artificial lighting on farms, such as using blue-free LED lights with an intensity below 10 lux (Asher et al., 2015), could induce higher endogenous melatonin synthesis in cows, potentially improving its immunomodulatory and protective effects on mammary gland health. Certainly, in this context, further studies are needed to further investigate this aspect, since most of the studies present in the literature have evaluated melatonin concentrations following manipulation of lighting conditions, as can be found in Table 1 (Asher et al., 2015; Romanini et al., 2019; Sahin et al., 2021; Teng et al., 2021).

Melatonin is also known to have positive effects on reproductive function in several animal species, particularly small ruminants. Indeed, melatonin administration has been observed in goats and sheep to improve reproduction, leading to increased progesterone synthesis, implantation rates and embryonic survival, and supporting follicular development (Saeedabadi et al., 2018; Tölü et al., 2022). Furthermore, melatonin implants use has become routine in both males and females, to stimulate oestrus and libido even during periods of the year that do not correspond to the physiological reproductive season of these species (Zarazaga et al., 2013, 2019; Tölü et al., 2022).

Similar results have also been observed in dairy cows, where, under heat stress conditions, melatonin supplementation during the dry period or prepartum phase can lead to a reduction in embryonic loss and, therefore, improved conception rates (Garcia-Ispierto et al., 2013; De Rensis et al., 2017; Morini et al., 2018).

Despite its positive effects on reproductive function described above, melatonin appears to have inhibitory effects on milk production. In this study, milk production was lower in the Night group (12.08 ± 0.96 kg/day) compared to the Day group (13.34 ± 1.05 kg/day). This effect may be due to physiological and environmental factors, but the possible role of melatonin in the secretion of prolactin, a hormone crucial to maintaining the lactogenesis process, cannot be overlooked: indeed, it is believed that melatonin can interfere with prolactin synthesis, as it has been observed that high melatonin concentrations result in a corresponding reduction in prolactin (Auldist et al., 2007). Furthermore, it has been shown that prolonged exposure to light, which leads to reduced melatonin synthesis, induces increased milk production in cows (Dahl et al., 2000). Therefore, to achieve a compromise between fertility and production, it may be of interest to conduct further studies to identify a threshold below which the positive effects of melatonin on the immune function of the mammary gland and reproductive system are maintained, but the negative effects on milk production do not become significant. In this way, optimal effects could be achieved in supporting both productivity and animal welfare.

Experiment 1 also evaluated the potential role of melatonin in regulating immune function in the mammary gland by evaluating SCC values and somatic cell immune component composition (DSCC) in milk samples from both groups. This study showed lower SCC values in milk from the Night group (129.71 ± 25.01 x10^3 cells/mL) compared to the Day group (196.39 ± 33.80 x10^3 cells/mL). These results are consistent with the observations of Asher et al. (2015) and other studies that observed a reduction in SCC in cows treated with exogenous melatonin, either via subcutaneous injection (4.6 or 9.3 mg/cow/day for four days), or via oral supplementation with rumen bypass (40–80 mg/cow/day) or edible capsules (120 mg/cow/day) (Yang et al., 2017; Yao et al., 2020; Wu et al., 2021; Li et al., 2024).

Furthermore, to evaluate the mechanisms of action through which melatonin exerts its immunoregulatory effect in the udder, we compared DSCC values between groups, thus differentiating the immune cell subpopulations present in the milk by quantifying the percentages of PMNs + lymphocytes and macrophages + epithelial cells. From this comparison, no significant differences emerged in the distribution of immune cell subpopulations between the Night and Day groups, as shown in Figure 5. The results of this study therefore suggest that the known immunomodulatory properties of melatonin (Andrani et al., 2024) may not be due to an effect of this hormone on somatic cell composition, as it appears unable to influence the proportions of cell types. The mechanisms by which melatonin exerts its protective role on mammary gland health may therefore more likely be due to an improvement in the local immune response.

Interest in melatonin has grown in recent years not only for its veterinary implications, but also for its potential benefits for human health. Indeed, it has been shown that consuming bovine milk naturally rich in melatonin increases blood melatonin concentrations, resulting in improved sleep quality and reduced anxiety (Valtonen et al., 2005; Bae et al., 2016; Milagres et al., 2014; Cohen Engler et al., 2012). In light of this, several countries already commercialize milk naturally rich in melatonin as a functional food, including Finland, Ireland, New Zealand, and the United States.

The characteristics and functions of melatonin highlight its importance not only as a modulator of immune function and circadian rhythms in dairy cow physiology, but also as a bioactive compound ingested through a functional food (milk) with effects on human health as well. From a One Health perspective, improving melatonin production through optimized dairy cow management could be of fundamental interest from both an animal health and welfare perspective and a human health perspective. The One Health approach recognizes the complex interconnection between human, animal, and environmental health, promoting integrated strategies to achieve optimal results in all three domains (Pitt and Gunn, 2024).

Therefore, improving melatonin synthesis and, consequently, increasing its content in milk can lead to improved animal welfare thanks to its immunomodulatory properties and the development of a highly functional, naturally derived food product that also benefits human wellness.

Finally, although further studies are needed to evaluate the possible long-term effects of increased melatonin production on mammary gland health in cows, the results of the present study seem to suggest that improved management of artificial light in the farm environment could lead to important positive implications both in terms of veterinary medicine and public health.

3.5 Conclusion

Experiment 1 confirms that melatonin release in bovine milk, similar to blood, follows circadian rhythms with higher concentrations in milk obtained from early morning milking. Furthermore, the reduction in SCC in the Night group and the absence of changes in somatic cell composition (DSCC) suggest that melatonin immunomodulatory effects are not due to an alteration in the distribution of immune cell subpopulations, but rather to an improvement in the local immune response. The study results therefore underscore the positive effect of melatonin on mammary gland health and suggest its potential protective effect against mastitis. This last aspect deserves further investigation with studies involving not only healthy but also mastitic animals.

In conclusion, focusing on the One Health approach, melatonin takes on value not only in terms of improving animal welfare, but also to produce a functional food that also has a positive effect on human health.

4. Experiment 2 – Integration of milk c-miRNAs and DSCC for stage-specific detection of mastitis

The work presented in this chapter has been published in Dall'Olio et al. (2025) – Differential expression of miR-223-3p and miR-26-5p according to different stages of mastitis in dairy cows, Biomolecules, 15(2), 235.

4.1 Introduction

Among the numerous miRNAs identified in bovine milk (previously reported in chapter 1.5), miR-26a-5p (miR-26) and miR-223-3p (miR-223) have emerged as two of the most consistently detected molecules. Both are involved in the regulation of immune and inflammatory pathways. In fact, although their specific roles in bovine mastitis are not yet fully elucidated, increasing evidence suggests that these miRNAs may participate in the fine-tuning regulation of immune responses under stress conditions, such as inflammation. The following chapter provides an overview of the known functions of miR-26 and miR-223.

miR-26 appears to play a role in regulating both immune and metabolic pathways. Although its role in mastitis in cattle has not yet been clarified, studies conducted in other animal species, such as donkeys and goats, suggest that miR-26 may be involved in the post-transcriptional regulation of genes involved in fatty acid metabolism, immune response regulation, and cell survival mechanisms (Fei et al., 2022). Specifically, miR-26 acts directly on molecules belonging to the PI3K-Akt and MAPK signalling cascades, which are known to regulate cell proliferation, apoptosis, and inflammatory responses. In mammary gland tissues, miR-26 appears to be able to confer resistance to apoptosis by modulating these signalling pathways, suggesting its possible cytoprotective role in cases of cellular stress or acute inflammation (Fei et al., 2022).

miR-26 has also been implicated in hyperglycaemic conditions, suggesting a further role in regulating metabolic stress. It can inhibit the expression of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 (PFKFB3), a regulator of glycolytic function, suggesting a potential role in modulating metabolism during immune activation (He et al., 2022). Furthermore, miR-26 is also associated with the regulation of the inflammatory response, as it appears to be involved in modulating the release of inflammatory mediators, such as cytokines and chemokines (Uttamani et al., 2023).

Despite its ubiquitous expression in the organism, miR-26 is highly abundant in mammary gland tissues and milk, being one of the most prevalent c-miRNAs detected in dairy cows (Tzelos et al., 2023).

To date, however, its role in bovine mastitis remains unclear, and available data in the literature are still limited. Some preliminary data have observed that miR-26 levels appear to exhibit alterations under physiological conditions. For example, in the early stages of pregnancy, miR-26 is overexpressed in the blood of cows, likely due to the immune, hormonal, and metabolic changes the body undergoes in this condition (Tzelos et al., 2023).

Furthermore, a correlation between changes in miR-26 expression and both CMT score, and lactation stage has been demonstrated, with higher c-miRNA concentrations in cows in the early stages of lactation and in those transitioning from a CMT score of 0 to 1, corresponding to an early inflammatory state (Tzelos et al., 2023). All this evidence supports the hypothesis that miR-26 could be indicated as a potential early biomarker of mastitis, as it appears to display alterations even before the onset of clinical signs.

It is known that miR-223 plays a key role in regulating the innate immune response: it regulates the proliferation, differentiation, and activation of PMNs (Johnnidis et al., 2008; Li et al., 2012) and modulates the polarization and functions of macrophages and dendritic cells (Jiao et al., 2021). Specifically, Jiao et al. (2021) observed that miR-223 promotes the differentiation of monocytes into M2 anti-inflammatory macrophages, associated with tissue repair and resolution of the inflammatory process. Furthermore, it appears to negatively regulate the inflammatory process by suppressing NF-κB activation and inhibiting the assembly and activation of the NLRP3 inflammasome, leading to a decreased production of cytokines, such as IL-1β and IL-18 (Yan et al., 2019; Valmiki et al., 2020; Jiao et al., 2021).

In bovine mastitis, overexpression of miR-223 has been demonstrated in mammary epithelial cells (MECs) and granulocytes, highlighting the involvement of this miRNA in the innate immune response (Jiao et al., 2022; Zhou et al., 2023).

The aim of Experiment 2 was to evaluate the expression patterns of four c-miRNAs previously associated with bovine mastitis, namely miR-26a-5p, miR-142-5p, miR-146a, and miR-223-3p.

The study was divided into two parts, *in vivo* and *in vitro*. In the first part, the four c-miRNAs were quantified in milk samples from cows in different stages of mastitis-induced inflammation to assess their potential as early biomarkers, correlating their expression with the inflammatory state of the mammary gland.

The second part evaluated the expression of the four c-miRNAs *in vitro* in the supernatant of monocultures of immune cells, specifically lymphocytes, PMNs, and monocytes, stimulated in a pro-inflammatory manner. This aimed to clarify their secretion dynamics and understand which cell types are most responsible.

Through *in vivo* and then *in vitro* evaluation, we attempted to shed further light on the role of these c-miRNAs in the pathophysiology of the mastitis process in cattle, supporting their potential as early and non-invasive biomarkers for the diagnosis of mastitis and for monitoring the possible inflammatory state of the mammary gland.

4.2 Materials and methods

4.2.1 Ethical statements

The Ethics Commission for Animal Experimentation (ECAE) of the University of Parma approved the Experiment 2 protocol (prot. 02/CESA/2025). The dairy cows included in this research were raised on private commercial farms and did not undergo any invasive procedures. Milk samples were collected exclusively during routine milking sessions. All samples collection and analyses were performed following established guidelines for milking recording and testing in dairy cattle (ICAR, 2023).

4.2.2 Milk sample collection and classification

Milk samples were obtained from 72 Italian Brown Swiss dairy cows, averaging 5.1 ± 1.6 years old, which were screened for SCC and DSCC. Based on PMN and lymphocyte percentages and the clinical status of the mammary gland, samples were categorized according to mammary inflammation. Two Liters of milk per cow were collected during evening milking sessions between February 2021 and April 2022 and immediately stored at 4°C. analyses were performed within 24 hours, after which samples were aliquoted into 2 mL Eppendorf tubes and frozen at -20°C for subsequent miRNA analysis.

Milk composition, including fat, protein, lactose, and casein content, was assessed using a MilkoScan FT3 (Foss Electric A/S, Hillerød, Denmark). SCC and DSCC (percentages of PMNs and lymphocytes) were measured with a Fossomatic 7DC (Foss Electric A/S, Hillerød, Denmark).

Following the validated procedure described by Zecconi et al. (2023), the 72 samples were divided into four groups of 18 samples each, based on the interaction between SCC and DSCC values. Using threshold values of 2×10^5 somatic cells/mL for SCC and 68.5% for DSCC, samples were classified as follows: cows with both SCC and DSCC below these thresholds were assigned to the control group (Ctrl); those with both values above thresholds formed the acute mastitis group (AM); animals with SCC above 2 × 10^5 cells/mL but DSCC below 68.5% were placed in the chronic mastitis group (CM); cows with SCC below 2 × 10^5 cells/mL and DSCC above 68.5% were identified as susceptible (SU) (see Figure 6).

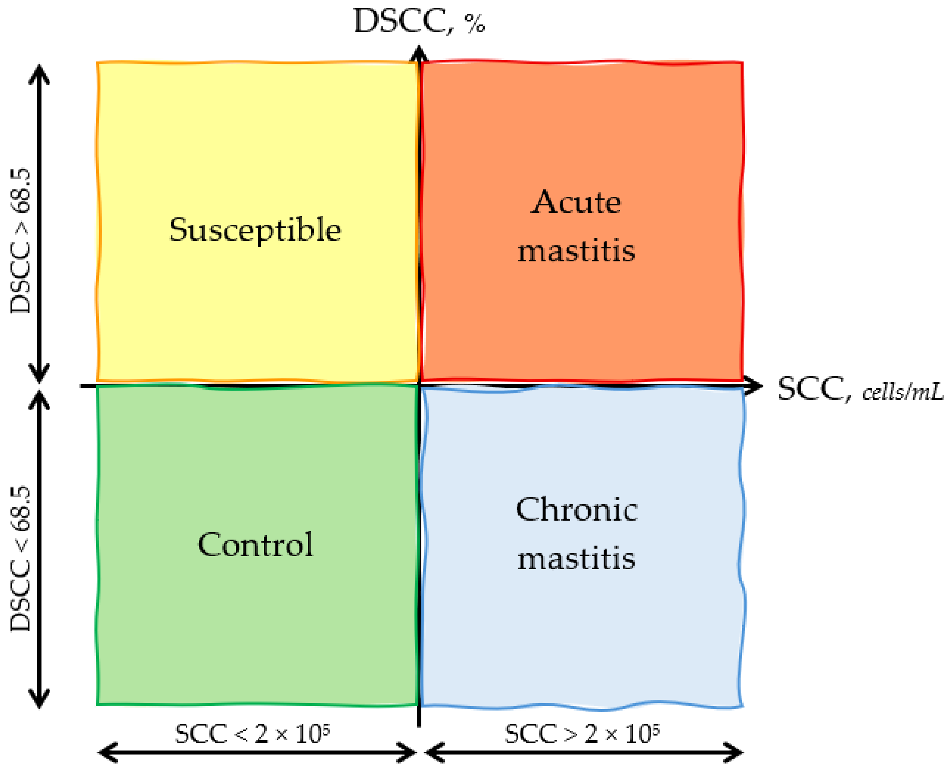


Figure 6 Schematic representation of the classification of milk samples into four groups based on combined somatic cell count (SCC) and differential somatic cell count (DSCC) thresholds. Samples were categorized as control (low SCC and low DSCC), susceptible (low SCC and high DSCC), acute mastitis (high SCC and high DSCC), or chronic mastitis (high SCC and low DSCC). The applied cut-off values were SCC < or ≥ 2 x 10^5 cells/mL and DSCC < or ≥ 68.5%.

4.2.3 Isolation, culture and inflammatory stimulation of bovine immune cells

Peripheral blood mononuclear cells (PBMCs) and PMNs were isolated from bovine blood collected at a slaughterhouse certified by the Italian Ministry of Health, in compliance with European Regulation (EC) 853/2004 (approval number CE-IT-218-M). PBMCs were isolated following the density gradient method with Histopaque-1.077® (Merck, Darmstadt, Germany), as described by Ferrari et al. (2013), while the PMN isolation protocol was adapted from Kouoh et al. (2000).

After isolation, PBMCs were incubated at 37°C with 5% CO_2 for 24 hours in cRPMI-1640 medium supplemented with 10% foetal bovine serum (FBS), allowing monocytes to adhere completely to the flask surface. Non-adherent cells were removed by washing twice with Phosphate Buffered Saline (PBS) solution. Monocytes were the incubated with 0.25% trypsin-EDTA (Gibco, Grand Island, NY, USA) for 5-10 minutes at 37°C and 5% CO_2. Trypsin activity was stopped by adding cRPMI-1640 + 10% FBS, followed by centrifugation and two additional washes in the same culture medium.

Lymphocytes isolation, following 24 hours of PBMCs incubation to allow monocytes adhesion, was performed by collecting the supernatant from the flask and washing it twice with cRPMI-1640 supplemented with 10% FBS. The isolated lymphocytes were then incubated at 37°C with 5% CO_2.

For PMNs isolation, 5 mL of EDTA-anticoagulated blood was layered onto Histopaque-1.077®. After centrifugation, the erythrocyte layer containing PMNs was collected. PMNs were isolated through erythrocyte lysis, obtaining a purity greater than 95%, as confirmed by differential blood cell counts on Diff Quick-stained smears. PMNs were incubated at 37°C with 5% CO_2 for 1 hour in cRPMI-1640 + 10% FBS to stabilize, considering their high sensitivity to even minimal mechanical stress (Dagur and McCoy, 2015).

Following incubation, immune cells were washed twice: lymphocytes and monocytes with cRPMI-1640 + 10% FBS, and PMNs with Hank’s Balanced Salt Solution (HBSS) without calcium, magnesium, and phenol red. Cells were then seeded at a density of 2 × 10^5 cells per well in 24-well plates. Cell viability, always above 95%, was verified using Trypan Blue exclusion test.

Lymphocytes were cultured either with 5 μg/mL phytoemagglutinin (PHA, derived from *Phaseolus vulgaris*; Sigma-Aldrich, Darmstadt, Germany) for 4 hours, or under control conditions in cRPMI-1640 medium supplemented with 10% FBS (Figure 7). Similarly, PMNs and monocytes were exposed to 1 μg/mL lipopolysaccharide (LPS, from pathogenic *E. coli* serotype 0111:B4; Merck, Darmstadt, Germany) for 4 hours or maintained in control medium (cRPMI-1640 + 10% FBS) without stimulation (Figure 7). At the end of the incubation period, the culture supernatants were collected from each well of the 24-well plates and immediately frozen at -20°C for subsequent Griess assay and miRNA analysis.

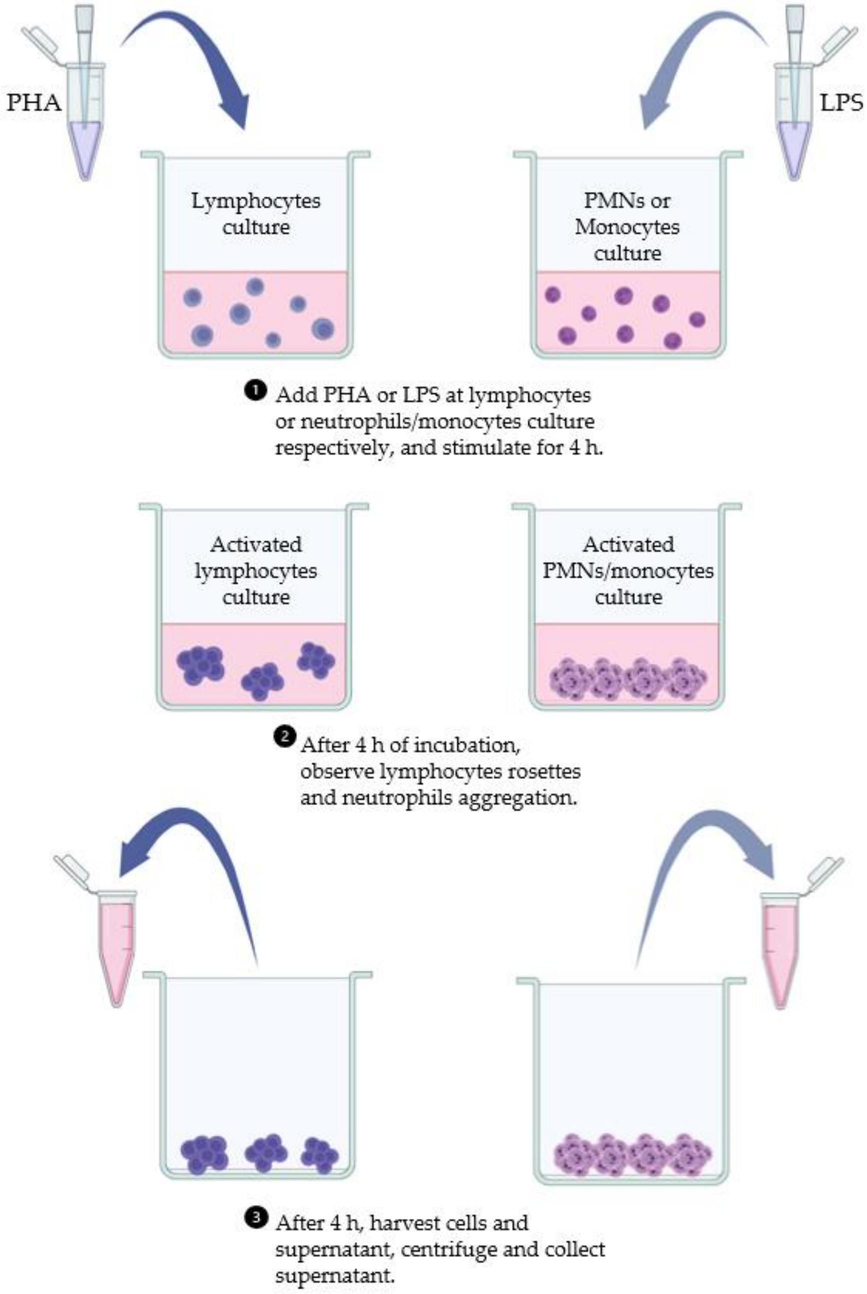


Figure 7 Schematic representation of the experimental workflow used to establish the inflammatory stimulation protocol in isolated immune cell subpopulations. Monocultures of polymorphonuclear leukocytes (PMNs), lymphocytes, and monocytes were prepared and subjected to targeted pro-inflammatory stimulation: lymphocytes were incubated with phytohemagglutinin (PHA), whereas PMNs and monocytes were exposed to lipopolysaccharide (LPS). All cell types were stimulated for 4 hours under controlled culture

conditions. The figure outlines the sequential steps of stimulation and subsequent processing aimed at collecting only the supernatant.

4.2.4 Measurement of nitrite as an indicator of nitric oxide production in immune cells

Nitric oxide (NO) production was indirectly assessed by measuring nitrite (NO_2^-) concentrations, a stable metabolic product of NO, in the culture medium of lymphocytes, monocytes, and PMNs, both under control conditions and following stimulation with PHA or LPS. Nitrite levels were determined after 4 hours of incubation performing the Griess reaction test, as reported by Saleri et al. (2022). Specifically, 100 µL of the culture supernatant was mixed with 100 µL of Griess reagent, freshly prepared by combining equal volumes of 1% (w/v) sulphanilamide in 5% (v/v) phosphoric acid and 0.1% (w/v) naphthylethylenediamine-HCl. The reaction proceeded for 15 minutes at room temperature. Absorbance was then measured at 540 nm using a VICTOR® Nivo™ Multimode Microplate Reader (PerkinElmer, Waltham, MA, USA). Nitrite concentrations were calculated based on a standard curve obtained in the culture medium using serial dilutions of sodium nitrite ranging from 0.39 to 50 µM ($R^2 = 0.99$).

4.2.5 Extraction and reverse transcription of c-miRNAs

A total of 72 animals were enrolled in this study, categorized based on the four previously described differential immune cell profiles in milk. C-miRNAs were isolated from milk samples using the Maxwell® RSC miRNA from Plasma and Serum Kit (Promega, Madison, WI, USA). This automated system, operated with the Maxwell® RSC Instrument (Promega, Madison, WI, USA), enables efficient extraction of high-quality total RNA with enhanced miRNA enrichment. For immune cell culture supernatants, c-miRNAs were extracted following a TRIzol® Reagent-based protocol (Life Technologies, Waltham, MA, USA) as reported by Ioannidis et al. (2018).

Immediately after extraction, total RNA (approximately 4 ng per sample) was reverse transcribed into complementary DNA (cDNA) using the miRCURY® LNA® RT Kit (Qiagen, Milan, Italy).

Reverse transcription reactions were performed using a StepOne™ thermocycler (Applied Biosystems, StepOne™ software v.2.3; Waltham, MA, USA) following the manufacturer instructions under the following thermal conditions: 42°C for 1 hour, followed by 5 minutes at 94°C. To monitor the efficiency and consistency of RNA extraction and reverse transcription, synthetic RNA spike-in controls were included: UniSp-2, UniSp-4, and UniSp-5 (RNA Spike-In Kit, For RT; Qiagen, Milan, Italy) during extraction, and UniSp-6 (miRCURY® LNA® RT Kit; Qiagen, Milan, Italy) during reverse transcription. The resulting cDNA samples were stored at -20°C until further analysis.

4.2.6 c-miRNAs expression analysis

The expression of c-miRNAs was assessed by real-time quantitative PCR (qPCR) using a StepOne™ thermocycler (Applied Biosystems, StepOne™ software v.2.3; Waltham, MA, USA). cDNA, previously diluted 1:30 in DNAse free water, was amplified in duplicate reactions with a final volume of 10 μL. Amplifications were performed using the miRCURY LNA SYBR® Green PCR Kit (Qiagen, Milan, Italy) along with specific primers targeting bovine sequences of miR-26a-5p (efficiency: 98.4%), miR-142-5p (efficiency: 98.6%), miR-146a (efficiency: 101.2%), miR-148a-3p (efficiency: 98.8%), and miR-223-3p (efficiency: 98.9%; Qiagen, Milan, Italy). Synthetic spike-in controls (UniSp-2, UniSp-4, UniSp-5, and UniSp-6; Qiagen, Milan, Italy) were included to monitor the efficiency of the reverse transcription and PCR processes.

The qPCR protocol consisted of an initial denaturation step at 95°C for 2 minutes, followed by 40 amplification cycles of 95°C for 10 seconds and 56°C for 60 seconds. A melting curve analysis was subsequently performed between 60°C and 95°C to confirm the specificity of the amplified products. Relative gene expression was calculated using the $2^{-\Delta\Delta Cq}$ method. Data were normalized to both the endogenous reference gene miR-148a-3p, which is highly expressed and stably present in bovine milk (Tzelos et al., 2022; Dall'Olio et al., 2025), and the exogenous control UniSp-2, following the approach described by Tzelos et al. (2022). For the interpretation of differential expression, a fold change threshold of ≥ 1.5 was considered indicative of upregulation, while values ≤ 0.67 were indicative of downregulation, in accordance with cut-off values commonly used in exploratory studies (Schaefer et al., 2010).

4.2.7 Statistical analysis

The sample size for the four groups of milk samples was estimated using the "pwr" package (version 1.3-0) within the R environment (version 4.3.3) and RStudio (release 2023.06.1). The calculation was performed assuming a significant level of 0.05, a statistical power of 80%, and a large, expected effect size, defined as 0.4.

Comparisons of c-miRNAs expression profiles among milk groups were conducted using the Kruskall-Wallis rank sum test, followed by pairwise comparisons with the Wilcoxon rank sum test, applying continuity correction and adjusting *p*-values with the Benjamini-Hochberg procedure.

The evaluation of c-miRNAs expression in immune cell cultures was performed using the Mann-Whitney test, the non-parametric equivalent of the independent t-test. The normality of data distribution was previously assessed using the Shapiro-Wilk test. Data obtained from the Griess assay (NO) passed the Shapiro-Wilk test for normality and were therefore analysed using one-way ANOVA, followed by Tukey's post hoc test to adjust for multiple comparisons.

4.3 Results

4.3.1 Milk composition and somatic cell parameters

Milk samples analyses conducted with the Fossomatic™ 7DC instrument (Foss Electric A/S, Hillerød, Denmark) yielded quantitative data on SCC and DSCC, which were used as a basis for dividing the 72 dairy cows into four experimental groups, as described in Chapter 4.2.2 of the Materials and methods section.

No significant differences were detected between groups when analysing the mean values of physiological parameters of cows included in the study, such as parity, days in milk (DIM), and daily milk yield.

Statistical analysis also revealed the absence of significant differences in milk composition, with details of the parameters evaluated reported in Table 2.

Furthermore, Table 3 provides details of the mean SCC and DSCC values for each experimental group, identified by the combined use of the SCC and DSCC cut-off previously described for distinguishing between a healthy and a mastitic cow.

Table 2 Mean values ± SD of physiological parameters (parity, days in milk and daily milk yield) and milk composition (fat, protein, casein and lactose) of experimental dairy cows, categorized by health status (n = 18 per group). Groups were healthy controls (Ctrl), cows susceptible to mastitis (SU), cows with acute mastitis (AM), and cows with chronic mastitis (CM). Physiological parameters provide information on lactation stage and production, while milk composition represents the main nutrient fractions. DIM = days in milk.

	Physiological parameters			Milk composition (%)			
Group	**Parity**	**DIM (d)**	**Milk yield (kg/sampling)**	**Fat**	**Protein**	**Casein**	**Lactose**
Ctrl	2.83 ± 1.34	184 ± 108	13.75 ± 4.81	4.06	3.70	2.95	4.85
SU	2.56 ± 1.34	205 ± 118	12.44 ± 3.79	4.08	3.72	2.96	4.84
AM	3.00 ± 2.14	181 ± 117	11.36 ± 4.38	4.08	3.78	2.98	4.65
CM	2.72 ± 1.93	217 ± 107	10.53 ± 3.84	4.39	3.98	3.15	4.62

Table 3 Mean values ± SD of somatic cell count (SCC) and differential somatic cell count (DSCC) of experimental dairy cows, divided by health status (n = 18 per group). The groups included healthy controls (Ctrl), cows susceptible to mastitis (SU), cows with acute mastitis (AM), and cows with chronic mastitis (CM). SCC provides an overall measure of somatic cell levels in milk, while DSCC represents the proportion of specific somatic cell types (polymorphonuclear leukocytes and lymphocytes).

Group	SCC (cells/mL)	DSCC (%)
Ctrl	$0.38 \times 10^5 \pm 0.01$	52.52
SU	$0.73 \times 10^5 \pm 0.02$	76.73
AM	$5.45 \times 10^5 \pm 0.30$	79.37
CM	$2.91 \times 10^5 \pm 0.62$	60.8

4.3.2 Expression patterns of selected c-miRNAs in milk samples

The relative expression of miR-26 was found to be significantly reduced in milk samples collected from cows classified as having acute mastitis (AM), chronic mastitis (CM), or identified as mastitis-susceptible (SU). Specifically, miR-26 expression was down-regulated by approximately 0.8-fold in both AM and CM groups ($p < 0.01$), and by 0.7-fold in the SU group ($p < 0.05$), when compared to the healthy control group (Figure 8A).

In contrast, miR-223 showed a marked up-regulation in cows experiencing AM. Its expression was significantly increased compared to all other groups, with fold change of 26.5 (vs. Ctrl), 24.4 (vs. SU), and 25.7 (vs CM), all with p-values < 0.01, as shown in Figure 8B.

Following an initial screening phase, two additional c-miRNAs, miR-142-5p (miR-142) and miR-146a (miR-146), were excluded from further analysis. These c-miRNAs did not exhibit significant differences in expression between the studied groups, suggesting a limited role in the milk-associated immune response under the tested conditions.

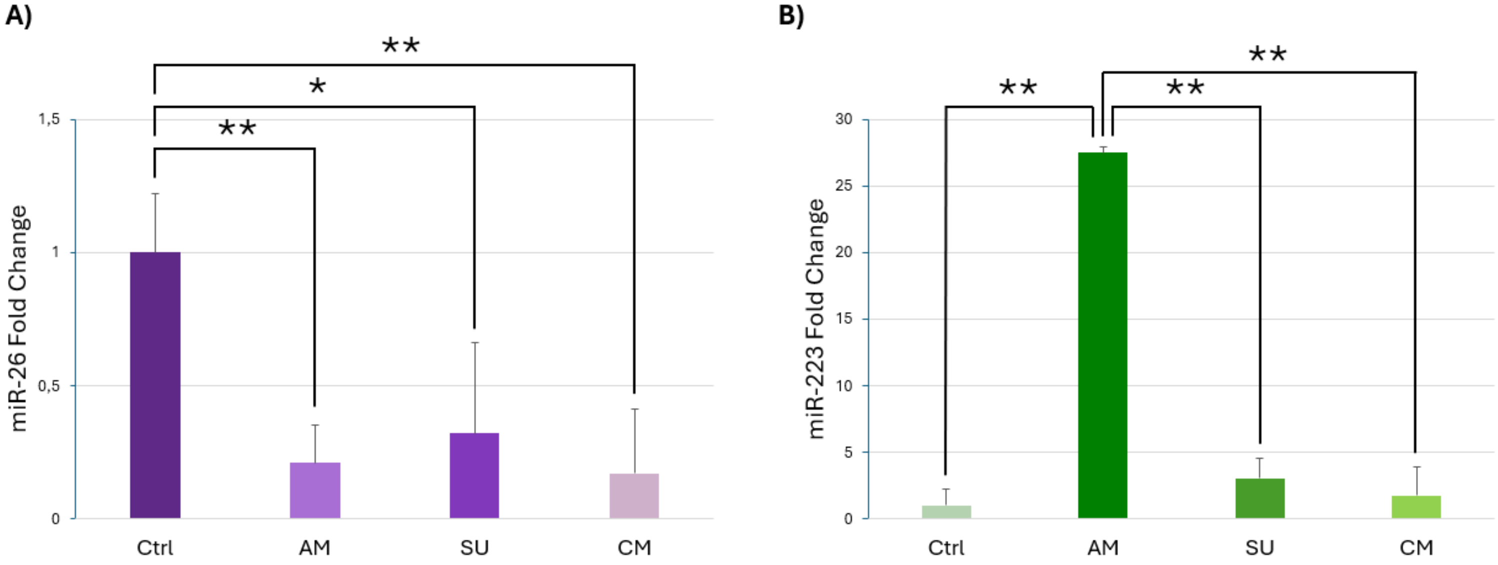


Figure 8 Relative expression of A) bta-miR-26a-5p and B) bta-miR-223-3p in milk samples divided by group based on udder health status. Groups are defined as follows (n = 18 per group): Ctrl, healthy control cows; SU, cows classified as susceptible to mastitis; AM, cows with acute mastitis; CM, cows with chronic mastitis. Data were analysed using the $2^{-\Delta\Delta Cq}$ method and normalized to the reference genes bta-miR-148a-3p and UniSp-2. The y-axis shows fold change relative to the control group. In panel A), SU, AM, and CM groups show lower expression of bta-miR-26a-5p compared with Ctrl. In panel B), bta-miR-223-3p expression is higher in the AM group compared with the other groups. * indicates a significant difference with *p*-value < 0.05; ** indicates a significant difference with *p*-value < 0.01.

4.3.3 Expression patterns of selected c-miRNAs in immune cells

The expression profile of c-miRNAs was evaluated in isolated immune cells population (lymphocytes, monocytes and PMNs) following in vitro stimulation with either PHA or LPS for 4 hours. Each group consists of samples derived from three independent experiments, with three replicates per experiment. Data were analysed using the $2^{-\Delta\Delta Cq}$ method, in which the expression levels of the gene have been normalized to the expression of the reference genes bta-miR-148a-3p and UniSp2. No significant variation of miR-26 expression was observed in lymphocytes or monocytes when comparing stimulated samples to their respective unstimulated controls (Figure

9A,B). In contrast, a significant downregulation of miR-26 was detected in PMNs treated with LPS, with expression levels reduced relative to the control (0.4-fold, $p < 0.01$), as illustrated in Figure 9C.

miR-223 expression showed a significant decrease in lymphocytes following 4-hour PHA stimulation compared to the unstimulated control group (0.2-fold, $p < 0.05$; Figure 9D). In contrast, neither monocytes nor PMNs exhibited significant changes in miR-223 expression upon LPS stimulation for the same duration (Figure 9E,F). However, a trend toward up-regulation was noted in monocytes cultures after LPS treatment.

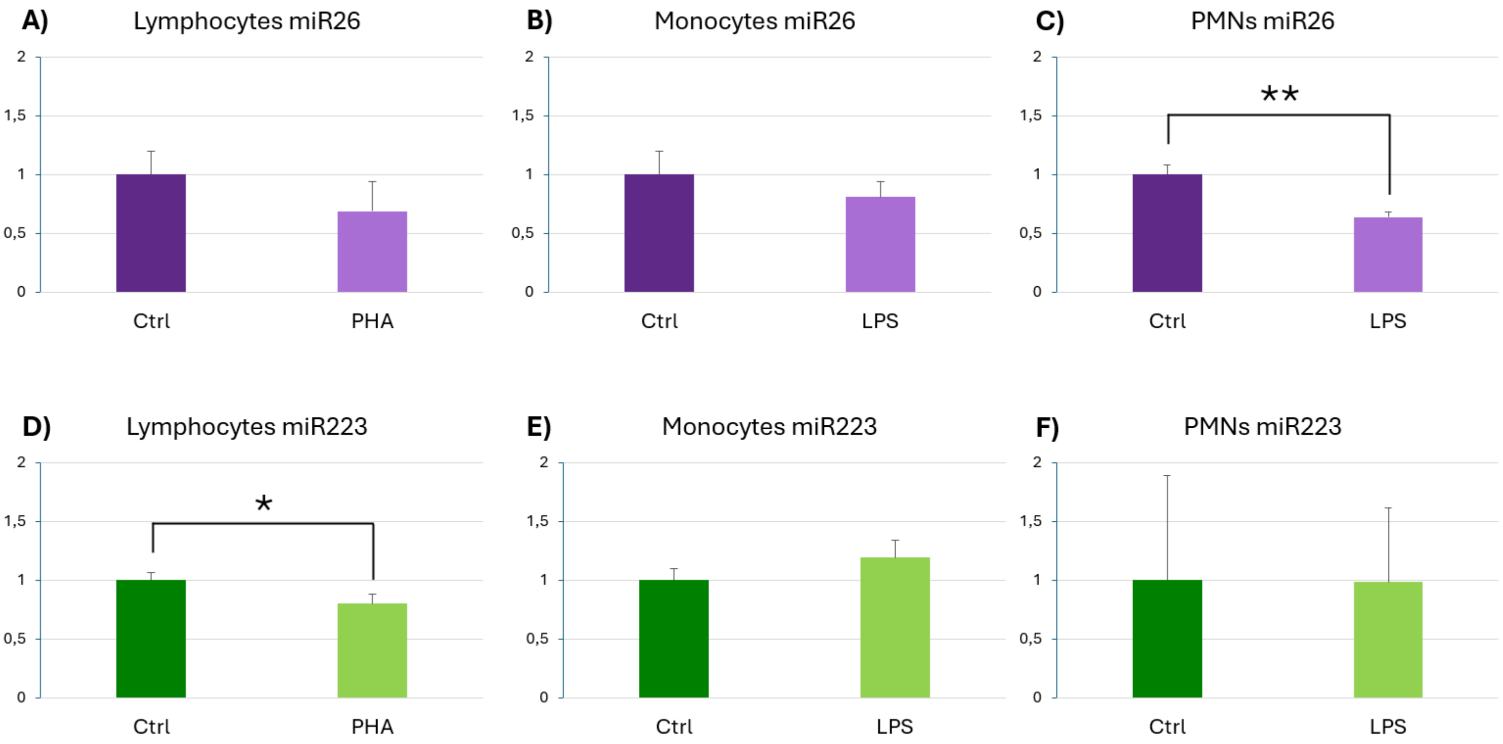


Figure 9 Relative expression of bta-miR-26a-5p and bta-miR-223-3p in culture media from isolated immune cell subpopulations under control and inflammatory stimulation conditions. Panels A-C show bta-miR-26a-5p expression in A) lymphocytes, B) monocytes and C) polymorphonuclear leukocytes (PMNs), whereas panels D-F show bta-miR-223-3p expression in D) lymphocytes, E) monocytes and F) PMNs. Lymphocytes were stimulated with phytohemagglutinin (PHA), while monocytes and PMNs were stimulated with lipopolysaccharide (LPS). Each group includes samples of three replicates from three

independent experiments. * indicates a significant difference with *p*-value < 0.05; ** indicates a significant difference with *p*-value < 0.01. Ctrl = control.

4.3.4 Nitric oxide response to pro-inflammatory stimulation in lymphocytes, monocytes and PMNs

As shown in Figure 10, results from the Griess assay revealed a significant increase ($p < 0.05$) in NO production across all immune cell type following *in vitro* stimulation. Specifically, after 4 hours of incubation with LPS or PHA, elevated levels of NO were detected in the culture supernatants of lymphocytes, monocytes, and PMNs compared to their respective unstimulated controls. These results indicate that both LPS and PHA can induce an inflammatory response characterized by enhances NO production, regardless of the immune cell subpopulation involved. An additional confirmation of the efficacy of the inflammatory stimulation protocol can be observed in the lymphocytes cultures. As early as 4 hours after PHA exposure, lymphocytes began to exhibit their characteristics rosette-like morphology, indicative of cellular activation, as reported by Wybran and Dupont (1982). This morphological shift is clearly visible in Figure 11.

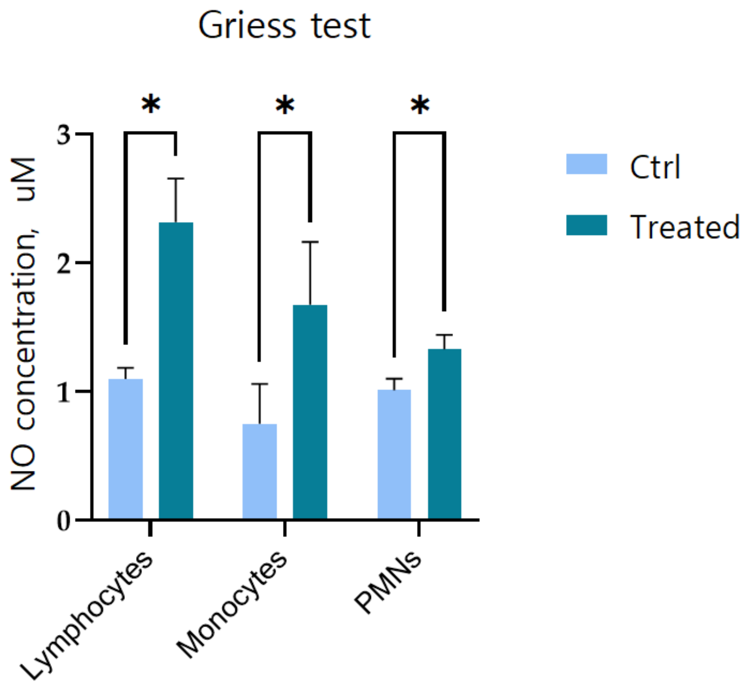
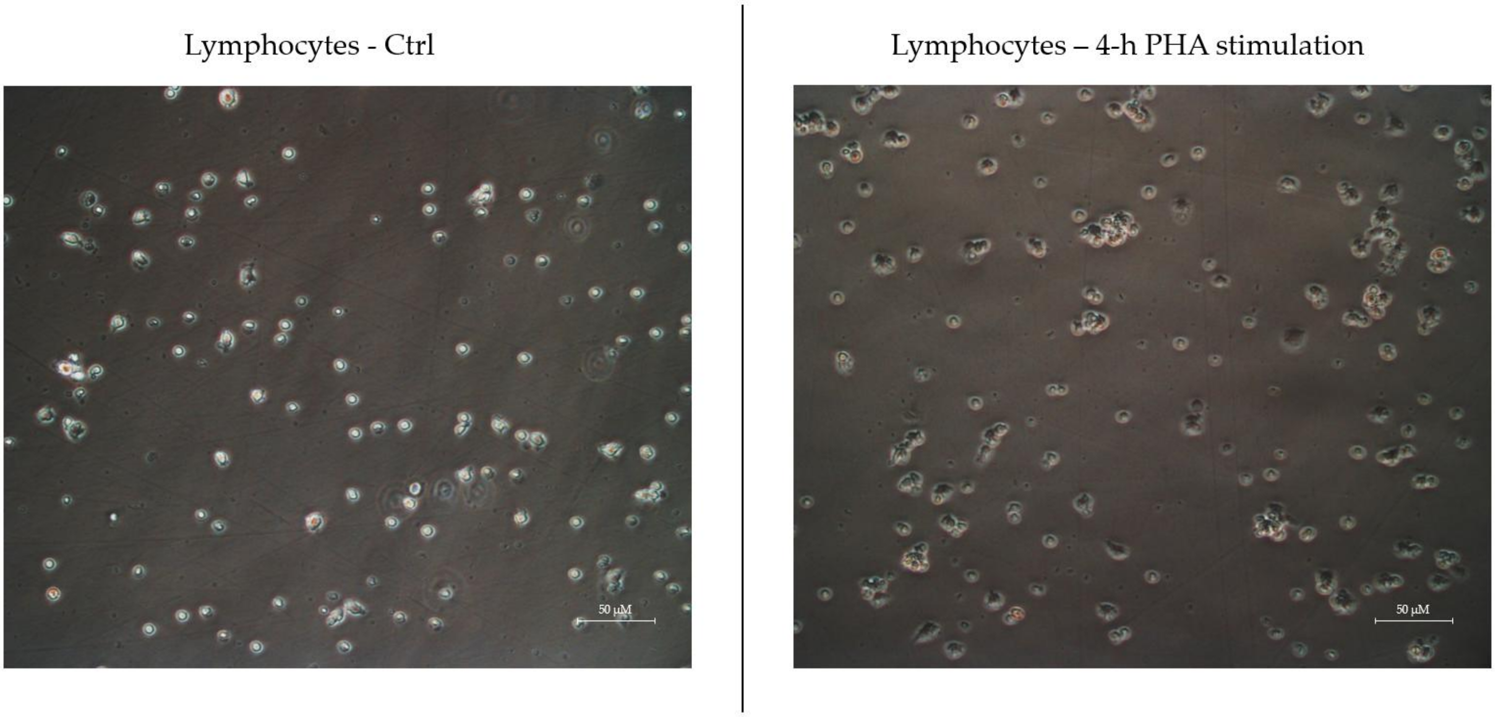


Figure 10 Nitric oxide (NO) concentration (mean ± SD) measured in the culture medium of lymphocytes, monocytes, and polymorphonuclear leukocytes (PMNs) after 4-hours stimulation with lipopolysaccharide (LPS) or phytohemagglutinin (PHA), compared with unstimulated control groups. In all cell types, treated cultures exhibited higher NO levels than their respective unstimulated controls. NO production following stimulation reflects activation of immune response, as LPS and PHA act as potent immunostimulatory agents. * indicates a significant difference with *p*-value < 0.05.


Lymphocytes - Ctrl

Lymphocytes – 4-h PHA stimulation

Figure 11 Microscopic images (20x magnification) of lymphocytes cultured at a density of 2 x 10^5 cell per well under control conditions (Ctrl) and after 4 hours of phytohemagglutinin (PHA) stimulation. Control lymphocytes display a homogenous spatial distribution with predominantly isolated cells, whereas PHA-stimulated lymphocytes exhibit early signs of activation, characterized by increased cell-cell interactions and the initial formation of inflammatory rosette-like aggregates.

4.4 Discussion

The aim of this study was to evaluate alterations in the expression of bta-miR-26a-5p, bta-miR-142-5p, bta-miR-146a, and bta-miR-223-3p in bovine milk in relation to different stages of mammary gland inflammation associated with the mastitis process. These four c-miRNAs were considered because they had already been associated with both the inflammatory process and the regulation of the immune response in the mammary gland (Tzelos et al., 2022). The study therefore aimed to evaluate whether their expression patterns could be associated with various inflammatory states of the udder and could indicate their potential as non-invasive and early molecular biomarkers for the detection of mastitis and acute inflammatory conditions affecting the mammary gland.

To this end, a study was conducted to comprehensively evaluate the behaviour of these c-miRNAs, both *in vivo* in milk collected in the field and *in vitro* in cultures of immune cells isolated from blood.

As reported in chapter 4.1, miR-26 is involved in immune regulation, acting on key signalling pathways such as PI3K-Akt and MAPK, and modulating processes related to apoptosis, inflammation, and cellular stress (Fei et al., 2022). Although its role in bovine mastitis is not yet fully understood, its high abundance in mammary tissues and milk, together with its altered expression under early inflammatory conditions, suggests a potential role as an early biomarker of mammary gland inflammation.

The results of the present study highlighted a significant downregulation of miR-26 expression in milk samples from the AM, CM, and SU groups; therefore, the alteration is evident in cows at different stages of mammary gland inflammation. These results are consistent with previous studies, which have demonstrated reduced miR-26 expression in inflamed tissues, which has been associated with increased cytokine production, alterations in cellular metabolism, and increased susceptibility to apoptosis (He et al., 2022; Uttamani et al., 2023).

To further investigate the mechanisms underlying its expression, miR-26 was then quantified in the supernatant of immune cells subjected to proinflammatory stimulation with LPS (PMNs and monocytes) or PHA (lymphocytes), a validated model of acute inflammation, as also supported by the results relating to the concentration of NO in the culture media (Figure 10). To the best of our knowledge, this is the first study to evaluate miR-26 (and miR-223) expression in the supernatant of bovine immune cells under inflammatory stimulation. PMNs already after 4 hours of stimulation showed a marked reduction in miR-26 levels, indicating a rapid response of this c-miRNA to changes in the immune system in presence of pro-inflammatory stimuli. PMNs are in fact the first components of the innate immune system to intervene in the event of infection, as they are the first to be called in situ by cytokines released by antigen-presenting cells (APC) that have previously encounter the pathogenic antigen (Kraus and Gruber, 2021). This rapid downregulation is a possible indicator that miR-26 is likely inhibited during the inflammatory process, allowing the transcription of inflammatory mediators normally repressed by this c-miRNA under physiological conditions.

Monocytes and lymphocytes showed a tendency toward reduced miR-26 expression. This suggests that miR-26 modulation likely occurs in all three cell types analysed, but at different times and magnitudes, depending on the cell type and its early or late involvement in the inflammatory response.

Experiment 2, along with the results of *in vivo* and *in vitro* studies, appears to support the hypothesis that reduced miR-26 expression may be an early biomarker of inflammatory activation in bovine mastitis. Its downregulation in all experimental groups except controls suggests a potential role for this miRNA as a negative regulator of inflammation, whose reduced expression could lead to an increased immune response even in the early stages of infection.

The characteristics observed to date of miR-223 (as reported in chapter 4.1) suggest its potential utility as a biomarker for inflammatory states, particularly due to its involvement in both the regulation of the inflammatory response and its resolution.

In the present study, miR-223 showed a significant increase in expression in the AM group, which was not observed in the CM and SU groups. These results suggest that miR-223 secretion into milk is dependent on the stage of the inflammatory process, with greater accumulation in milk during the acute, rather than initial, phase of inflammation. This evidence is also supported by the results obtained from the *in vitro* phase of the study. No differences were found between the treated (4-hour stimulation) and control groups for all cell types examined, highlighting that miR-223 does not exhibit changes in expression in the early stages of inflammation.

These results are consistent with the observations of Srikok et al. (2020), who observed increased levels of miR-223 in cows with mastitis, with a greater increase in milk than in blood samples. This could indicate a certain degree of tissue specificity of this miRNA and suggest milk as the preferred matrix for its quantification. Furthermore, Tzelos et al. (2022) demonstrated that whole milk is more suitable than skimmed milk for the quantification of miRNAs, as they may be found in milk associated with fat globules.

Thus, our study revealed downregulation of miR-26 at all stages of the mammary gland inflammatory process and its early alteration *in vitro*, suggesting its potential utility as an early biomarker for mastitis. In contrast, miR-223 showed a marked increase only in the AM group, suggesting its potential role as a marker in conditions of acute inflammation, as evidenced by *in vitro* results, in which its expression was not altered following short-term proinflammatory stimulation.

These results seem to support the hypothesis that differential assessment of miR-26 and miR-223 expression in combination with already established parameters for the diagnosis of mastitis, such as SCC and DSCC, could improve diagnostic reliability. This approach could allow for the distinction between animals in the very early stages of inflammation, those with chronic inflammation, and those with acute inflammation, also allowing for monitoring the progression or regression of the disease.

From an operational perspective, this study proposes a diagnostic decision-making model illustrated by the flowchart shown in Figure 12. This flowchart integrates clinical observations, miR-26 and miR-223 expression patterns, and DSCC to distinguish between the different stages of mastitis.

The diagram outlines an integrated diagnostic strategy that combines the differential expression of miR-26 and miR-223 with DSCC values and the presence or absence of clinical signs. The flowchart is designed to guide the classification of cows into four categories: healthy (control group), susceptible (early inflammation), chronic mastitis, and acute mastitis. Downregulation of miR-26 is indicative of early inflammatory states, whereas upregulation of miR-223 is associated with acute mastitis. DSCC thresholds serve as an additional discriminating factor to enhance diagnostic resolution.

This process is also non-invasive, as it is based exclusively on the analysis of milk samples, and could be integrated into routine cow health monitoring protocols on farms.

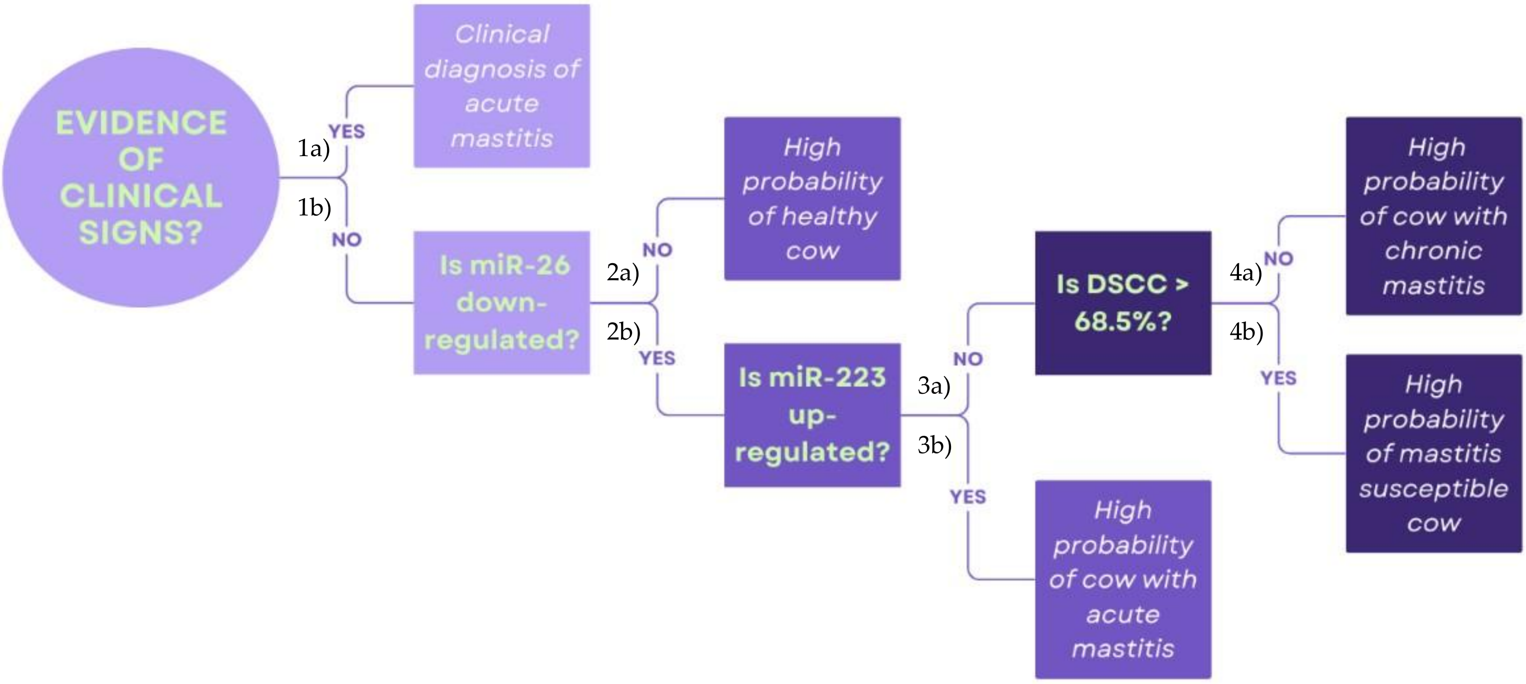


Figure 12 Diagnostic decision-making flowchart for the identification and classification of mastitis stages in dairy cows based on molecular profiling. This approach integrates molecular and cytological markers and aims to improve the sensitivity and specificity of mastitis detection using non-invasive milk-based biomarkers. The first step assesses the presence of clinical signs. 1a) If they are observed, the cow is clinically diagnosed with acute mastitis. 1b) If no clinical signs are detected, the expression level of miR-26 is assessed. 2a) When miR-26 is not downregulated, the cow is classified as having a high probability of being healthy. 2b) If miR-26 is downregulated, miR-223 is evaluated. 3a) If miR-223 expression is not upregulated, it leads to the evaluation of the DSCC parameter. 3b) When miR-223 is upregulated, the model indicates a high probability of acute mastitis. 4a) A DSCC value lower than 68.5% indicate a high probability of chronic mastitis. 4b) If DSCC is higher than the 68.5% threshold, the cow is

considered highly susceptible to mastitis. miR-26 = bta-miR-26a-5p; miR-223 = bta-miR-223-3p; DSCC = differential somatic cell count.

4.5 Conclusion

The results of this study suggest miR-26 and miR-223 as potential new non-invasive biomarkers for differential screening of milk samples and characterizing the different phases of the inflammatory process associated with mastitis in dairy cows. These initial *in vivo* findings were supported by the *in vitro* phase of the study, which revealed cell-type-specific responses to inflammatory stimuli, highlighting early variations in miR-26 and later variations in miR-223.

The differential analysis of these two miRNAs, when combined with traditional parameters such as SCC and DSCC, may therefore offer a new diagnostic approach capable of distinguishing between early, chronic, and acute inflammatory states. This is of particular interest given the limitations of routinely used methods, which often yield false-positive or negative results and show insufficient sensitivity for detecting the early or subclinical stages of mastitis in dairy cows.

5. Overall conclusions

The two research approaches presented in this thesis, relating to melatonin content in milk and the expression of miR-26 and miR-223, have the common goal of improving udder health monitoring and mastitis management in dairy cows.

Experiment 1 showed a significant increase in melatonin content in the Night group, which, combined with the decrease in SCC, suggests a potential immunomodulatory and protective effect of melatonin on mammary gland health. Considering that the DSCC data revealed no differences between the two groups, the mechanism by which melatonin affects somatic cells does not imply a change in their composition but is likely due to an enhancement of immune function through the modulation of oxidative stress and inflammatory mediators. This identifies melatonin as a possible natural, endogenous, and inducible factor that can contribute to mammary gland homeostasis and mastitis prevention.

Experiment 2 revealed differential expression of miR-26 and miR-223 in relation to the inflammatory stages associated with mastitis. Specifically, miR-26 showed early involvement in inflammatory processes, also considering its ability to enhance innate immune responses. MiR-223, on the other hand, showed more marked but late variations, related to its involvement in the acute or recovery phases of the inflammatory process.

This study also revealed that both c-miRNAs considered are actively secreted by PMNs, lymphocytes, and monocytes, confirming their immune origin and increasing their relationship with inflammatory processes within the alveolar microenvironment.

Following these two studies, it is possible to hypothesize a complementary role between endocrine and molecular components in managing mammary gland health. Melatonin may contribute to the prevention of inflammatory conditions, while miR-26 and miR-223 appear to be potential molecular biomarkers for early and non-invasive diagnosis of mastitis. The highlighted relationship with SCC and DSCC underscores the practical implications of the study: the combined use of molecular parameters with traditional indicators of breast health may lead to improved diagnosis and management of mastitis.

6. Future perspectives and practical implications

The combined analysis of melatonin content and c-miRNA expression patterns in milk lays the foundation for the development of more sensitive, reliable, and informative tools for monitoring bovine mastitis.

Bovine mastitis monitoring, from an applied perspective, could be of particular interest as a parameter for making farm management decisions, such as assessing animal housing conditions. Given the inducible nature of melatonin following optimal artificial lighting management, it could be interesting to evaluate it as a selective trait for mastitis resistance. Therefore, genetically selecting those animals that achieve higher production peaks under the same conditions could help further strengthen the species' resistance to mastitis.

From a practical perspective, the identification of miR-26 and miR-223 as potential diagnostic biomarkers for mastitis could be the first step toward the creation of rapid molecular diagnostic kits that can be used directly by farmers in the field. These kits can not only distinguish between healthy and mastitis-affected animals but also identify the different stages of inflammation. This would not only allow for rapid intervention but also reduce the use of antibiotics, reducing the overall impact of mastitis on animal welfare, dairy production, and public health, thus contributing to the primary objectives of One Health.

In both areas of research, further studies are certainly needed to potentially consolidate the results on larger and more diverse samples, considering variables such as lactation stage or breed. Furthermore, it will be crucial to fully understand the mechanisms through which melatonin is able to perform its function in regulating the immune response.

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