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## Effects of melatonin treatment on milk traits, reproductive performance and immune response in Sarda dairy sheep

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### ABSTRACT

In ruminants, the role of melatonin in the control of reproductive seasonality is well reported, but it is still little known about its action on milk traits and on the immune system. The aim of this study was to assess the effect of melatonin on milk yield and composition, somatic cell count (SCC), some cytokine blood concentration and reproductive resumption in sheep. One hundred lactating sheep were allocated to two groups (of 50 sheep each), M (treated with melatonin) and C (controls), and exposed to the rams for 50 d. Time period in days from ram introduction to lambing (TRIL) and litter size were recorded. Every 15 d, from 1 March to 30 April, the individual daily milk yield was registered and milk composition and SCC, were analysed. The levels of Interleukin 2 and 6 (IL-2 and 6) and tumour necrosis factor- $\alpha$  (Tnf- $\alpha$ ) were evaluated every 15 d. The highest fertility rate ( $p < .01$ ) and the shortest TRIL ( $p < .05$ ) was recorded in M group. Milk yield and composition were similar between groups. The somatic cells decreased continuously in the treated ewes ( $p < .01$ ) and increased continuously in untreated ewes ( $p < .05$ ). IL-2 and IL-6 increased in M group, although no statistical differences were found between groups. Melatonin administration improved reproductive efficiency, did not affect milk yield and composition and decreased somatic cells. This last effect could help to maintain healthy mammary gland and encourage farmers to reduce the use of drugs against mastitis.

### HIGHLIGHTS

- Melatonin is involved in different physiological processes, such as the control of reproductive seasonality, milk production and the immune response.
- Melatonin administration in Sarda sheep did not modify milk yield and composition, decreased SCC and advanced reproductive resumption in spring.
- Melatonin could be used to strengthen the sheep immune response in order to reduce the use of drugs for both therapeutic and preventive purposes against mastitis.

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Melatonin treatment; reproductive activity; milk traits; SCC; Sarda ewes

## Introduction

Melatonin is considered a multitasking molecule for the wide variety of effects, such as the control of circadian and circannual rhythms, including the seasonal control of reproduction, and for its antioxidant and anti-inflammatory activity (Reiter 1991; Tan et al. 2003). The control of reproductive seasonality in small ruminants is influenced by the photoperiod trend through the melatonin secretion (Bittman et al. 1983). Daily variations in melatonin secretion synchronise the endogenous rhythm of reproduction to the external environment through control of gonadotropin-releasing hormone (GnRH) and luteinising hormone (LH)

secretion (Barrell et al. 1992). This synchronisation ensures that the lambing occurs in the most suitable period for the survival of the offspring. Spring lambing is essential for wild species, but it also causes some problems in farmed animals. The seasonal milk and meat production, in fact, causes variations in the price paid to producers and also in the availability of fresh animal products (Chemineau et al. 2008). The use of exogenous melatonin has received much attention in different countries in order to control reproductive seasonality (Abecia et al. 2011). Melatonin treatments significantly advance the reproductive season and reduce the length of lambing period in sheep,

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resulting in improved flock management (Mura et al. 2019, 2017).

In sheep and goats, however, melatonin administration or the simulation of short photoperiods, caused the decrease in milk yield and in fat, protein and lactose concentrations (Garcia-Hernandez et al. 2007; Molik et al. 2011). In cattle, the administration of melatonin influenced milk fat synthesis and led to a decrease in somatic cells, presumably due to the effect of this hormone on the immune system (Yang et al. 2017; Wang et al. 2019). The role of melatonin in the inflammatory processes is to regulate the activation of the immune system through the secretion of cytokines (Pierpaoli and Maestroni 1987). Subclinical mastitis is a major concern for the dairy industry as it causes important economic losses (Tedde et al. 2019). The rise in SCC, that is common during mastitis, causes the release of many chemotactic factors, as inflammatory mediators, such as cytokines (Alluwaimi 2004). Among them, IL-2 and IL-6 and Tnf- $\alpha$  play a central role in immunoregulation by stimulating proinflammatory activity with accumulation of leukocytes at the site of infection (Alluwaimi 2004). Experimental and clinical data display that melatonin is able to modulate both pro- and anti-inflammatory cytokines in different pathophysiological conditions (Mauriz et al. 2013; Yu et al. 2017; Tarocco et al. 2019). Furthermore, in goats, feeding supplementation with food rich in melatonin, such as *Zea mays* seed, increased endogenous melatonin concentration and IL-2 and IL-6 levels with associated decrease of Tnf- $\alpha$  (Singh and Halder 2017). So, the aims of this study were: to investigating the effect of melatonin treatment (1) on milk yield and composition, SCC, blood levels of cytokines (IL-2, IL-6 and Tnf- $\alpha$ ) and (2) on reproductive activity in Sarda sheep.

## Materials and methods

### *Animal management and reproductive design*

Animals used in this research were managed and treated by the farm's veterinarian in accordance with the European Commission recommendation. The melatonin treatments made in this study are common techniques carried out in sheep farms in order to optimise their reproductive management. Milk and blood samples were collected by the National Health Veterinary Service during the routine procedures of the flock health management protocols. Sheep were included in this trial with the agreement of the farmers.

The study was conducted in 2018 on a farm located in North Sardinia (40.70 N), raising approximately 800

dairy sheep of Sarda breed. During the day the animals grazed on leguminous and gramineous grasses and during the night the sheep were penned and received hay (11.1% raw protein and 7.2 MJ ME/kg DM) and water *ad libitum*. At the time of the two daily milking the ewes received also 400 g of commercial concentrated food per head (20.4% of raw protein and 12.5 MJ ME/kg of DM). For the research, 100 lactating sheep, aged 3–5 years ( $3.2 \pm 0.6$  years), with parity number 2–4, with body condition score (BCS) 2.5–4.0 ( $3.3 \pm 0.4$ ), and with a single lamb born between 20 December 2018 and 10 January 2019, were chosen. The lambs were weaned on 20 February, and the ewes were milked twice a day, at 6.00 a.m. and at 5.00 p.m. On March 1, the 100 animals were randomly distributed into two equal groups of 50 ewes each (M and C), based on the date of lambing, parity number, age and the milk production levels. On March 1, group M received one subcutaneous implant (18 mg) of melatonin (Melovine, CEVA Salute Animale, Agrate Brianza, MB) while group C was not treated. After treatment, the two groups were separated. On April 5, in both groups three rams were introduced, and removed after 50 d. All the rams were provided with marker harnesses, so that matings were registered daily. Marker harnesses were changed every 10 d. From 45 to 90 d from the ram introduction, gestation was diagnosed by transabdominal ultrasound examination through the Tringa Esaote equipment (Esaote Europe BV, Maastricht, Netherlands) equipped with a 5 multi-frequency linear probe, 0–7.5 MHz. From September 2, the lambing date and numbers of newborn lambs were recorded. Fertility rate (the ratio between the number of lambed ewes and the ewes exposed to the rams), litter size (number of newborn lambs per lambed ewes) and time period in days from ram introduction to lambing (TRIL) were calculated. The onset of reproductive activity in the two groups was calculated through TRIL.

### *Milk sampling and analysis*

From 1 March to 30 April, every 15 d, the individual daily milk yield was recorded. On the same dates, individual samples of 50 mL of milk were taken during morning milking by the milking machine and were kept refrigerated at 5 °C until reaching the laboratory where they were analysed. Individual milk samples were used to analyse the percentage of fat, protein, lactose and somatic cell count (SCC). The daily milk yield (kg/day) was the sum of morning and afternoon milking yields. Milk fat, protein and lactose contents

were measured using a Milkoscan FT 6000 FOSS milk analyser (Foss Electric A/S, Hillerød, Denmark), according to FILIDF recommendations (ISO 9622:2013; ISO-IDF, 2013). SCC was determined with a Fossomatic 5000 FOSS somatic cell counter according to ISO 13366/IDF148 (2006). In order to normalise the distribution of SCC, it was transformed in its logarithmic score, somatic count score ( $SCS = \log_2 (SCC/100) + 3$ ) (Ali and Shook 1980).

### Blood sampling and analysis

From March 1 to April 30, every 15 d individual blood samples (10 mL) were collected from the jugular vein by tube with Lithium Heparin as an anticoagulant (VACUTEST KIMA, Arzergrande, PD, Italy). Blood samples were transported to the laboratory refrigerated at 5 °C and, within 1 h of collection, they were aliquoted and frozen at -20 °C until the time of analysis. From each sample the levels of IL 2 and 6, and of Tnf- $\alpha$  were evaluated. The samples were centrifuged at 3000 rpm for 20 min and plasma was collected and, then, stored at -20 °C until use. The measurement of IL-2, IL-6 and Tnf- $\alpha$ , were made using sheep commercial ELISA kits (Genorise, Scientific, Inc., Glen Mills, PA) according to manufacturer's protocol. Results are given as pg/mL, detection range and assay sensitivity were 78–5000 and 15 pg/mL, respectively.

### Statistical analysis

R statistical software version 4.0.0 R Core Team 2020R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL <https://www.R-project.org/> was used to analyse the associations between the treatment and the reproductive activity, measured as fertility rate, litter size and TRIL. To compare the differences in lambing between treatment and control groups every 10 d a chi-square test was used. The differences in number of mated or not mated, in pregnant or not pregnant and in lambing or not lambing between the treated and untreated groups were evaluated using chi-square test for independence (categorical variables). To analyse TRIL and litter size, was performed the following linear model:

$$y_{jkmno} = \mu + T_j + P_k + A_m + S_n + e_{jkmno}$$

where  $y_{jkmno}$  is the trait measured for each animal,  $\mu$  is the overall mean,  $T_j$  is the fixed effect of the treatment ( $j=2$  levels, 0= melatonin group, 1= control group),  $P_k$  is the fixed effect of parity number ( $k=3$  levels, 0=2parity, 2=3 years, 5=4 years),  $A_m$  is the

fixed effect of the sampling ( $m=5$  levels, from 1 to 5),  $S_n$  is the random effect of ewe ( $k=1-100$  levels) and  $e_{jkmno}$  is the standard error. The differences in cytokines concentration, milk yield, milk composition and SCS between the two groups were statistically analysed using linear mixed-effects model by lme4\_4.0.2 R Package (Bates et al. 2015), where the period of the study (5 levels), melatonin treatment (2 levels) and parity number (3 levels), were set as fixed effects, while ewes (1–100) were set as random effects. For each analysis, statistical significance was considered if  $p \leq .05$ .

### Results

The highest number of lambing ewes was recorded in the M group ( $p < .01$ ) compared to C (Table 1). The same trend among groups was recorded also for the number of mated, pregnant and empty ewes. A difference of about 2% was reported between the number of mated and pregnant and between pregnant and lambing ewes ( $p < .01$ ). The lowest number of empty ewes was recorded in the M group ( $p < .01$ ). Fertility rate was higher in the M group compared to C ( $p < .01$ ) (Table 2). The TRIL was lower in the M compared to the C group ( $p < .05$ ). Litter size showed no differences between groups. The treated ewes lambing approximately 10 d earlier than controls. On the 161st and 170th day after the ram introduction the group M showed a higher number of lambing ewes compared to the control group ( $p < .01$ ). In the treated group, the lambing peak was recorded at day 161–170, while in the control group at day 171–180 from ram introduction (Table 3).

Milk yield showed a similar trend in both treated and control groups, with an increase (without

**Table 1.** Total number, mated, pregnant, lambing and empty ewes, in M and C groups.

Group	Total n.	Mated	Pregnant	Lambing	Empty
M	50	48 <sup>B</sup>	47 <sup>B</sup>	46 <sup>B</sup>	4
C	50	37 <sup>A</sup>	36 <sup>A</sup>	35 <sup>A</sup>	15

M: melatonin treated group; C: control group (without melatonin treatment). Different capital letters in columns differ significantly for  $p < .01$ .

**Table 2.** Number of ewes, fertility rate, litter size and TRIL in M and C groups.

Group	Total n.	Fertility rate (%)	Litter size	TRIL (days)
M	50	92 <sup>A</sup>	1.12 ± 0.15	170.91 ± 9.23 <sup>a</sup>
C	50	70 <sup>B</sup>	1.18 ± 0.12	180.15 ± 10.67 <sup>b</sup>

M: melatonin treated group; C: control group (without melatonin treatment). TRIL: time period in days from ram introduction to lambing; Different capital letters in columns differ significantly for  $p < .01$ ; Different lowercase letters in columns differ significantly for  $p < .05$ .

statistical significance) in the 3rd and 4th sampling compared to the others (Table 4). The concentration of milk fat, protein and lactose remained stable over the observed periods in both groups. The somatic cells, instead, showed a continuous decrease in the treated animals from the first to the fifth sampling ( $p < .01$ ). Instead, in untreated animals there was a continuous increase from the first to the fifth sampling ( $p < .05$ ). The comparison between groups showed that the SCS exhibited a difference in the third, fourth and fifth sampling for  $p < .01$  (Figure 1). Parity number did not affect SCS. Finally, the analysis of cytokines in the treated group displayed an increase in circulatory IL-2 and IL-6 level while TNF- $\alpha$  maintained similar levels in both groups (Figures 2–4). The comparison between treated and control groups has not shown statistically significant evidence.

### Discussion

Treatment with melatonin improved reproductive efficiency in Sarda sheep and this result is in agreement with our previous experiences in adult and ewe lambs. (Luridiana et al. 2016; Mura et al. 2017). Moreover, this data are in accordance with what found in other European sheep breeds with some differences related to the treatment period and/or to the breed (Abecia et al. 2007). Another important aspect is the higher concentration of lambing in treated ewes compared to controls, as evidenced by the TRIL. This is an important result, as reported by Carcangiu et al.

**Table 3.** Number of ewes lambing from 150th to 200th day after ram introduction (every 10 d) in the treated (M) and control (C) groups.

Group	Days from ram introduction to lambing				
	150–160	161–170	171–180	181–190	191–200
M	3	22 <sup>B</sup>	13	4	2
C	1	4 <sup>A</sup>	14	8	8

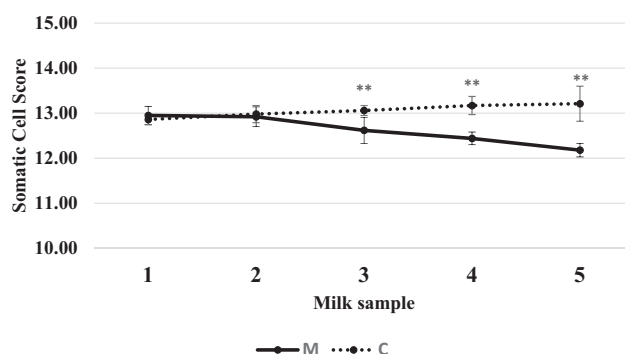
M: melatonin treated group; C: control group (without melatonin treatment). Different capital letters in columns differ significantly for  $p < .01$ .

**Table 4.** Least square means  $\pm$  SEM and  $p$  value for milk yield and milk composition (fat, protein and lactose content) of each level of the fixed effects (treatment, parity number and sampling).

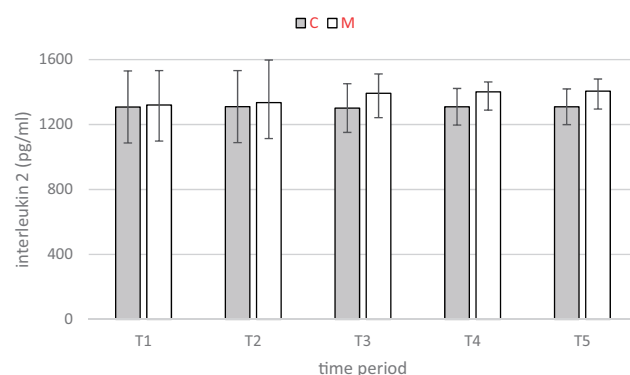
Factor	Level	Yield (g/die)	$p$ Value	Fat (%)	$p$ Value	Protein (%)	$p$ Value	Lactose (%)	$p$ Value
Treatment	M	1764 $\pm$ 201	.152	6.37 $\pm$ 0.7	.625	5.17 $\pm$ 0.2	.109	4.86 $\pm$ 0.5	.729
	C	1734 $\pm$ 219		6.36 $\pm$ 0.6		5.02 $\pm$ 0.3		4.87 $\pm$ 0.3	
Parity number	2	1743 $\pm$ 208	.214	6.49 $\pm$ 0.3	.087	5.05 $\pm$ 0.3	.153	4.86 $\pm$ 0.4	.547
	3	1731 $\pm$ 199		6.38 $\pm$ 0.9		5.12 $\pm$ 0.5		4.89 $\pm$ 0.3	
	4	1778 $\pm$ 220		6.28 $\pm$ 0.7		5.11 $\pm$ 0.1		4.83 $\pm$ 0.5	
Sampling	1	1645 $\pm$ 219	.511	6.43 $\pm$ 0.4	.221	5.05 $\pm$ 0.6	.334	4.83 $\pm$ 0.5	.447
	2	1745 $\pm$ 218		6.32 $\pm$ 0.7		5.07 $\pm$ 0.7		4.84 $\pm$ 0.3	
	3	1803 $\pm$ 196		6.22 $\pm$ 0.4		5.06 $\pm$ 0.2		4.84 $\pm$ 0.1	
	4	1823 $\pm$ 211		6.36 $\pm$ 0.8		5.12 $\pm$ 0.4		4.94 $\pm$ 0.6	
	5	1728 $\pm$ 214		6.50 $\pm$ 0.5		5.17 $\pm$ 0.3		4.86 $\pm$ 0.2	

$p$  Value  $< .05$  was considered statistically different.

(2012), since it allows the rationalisation of the farm work in the management of lambing, weaning and the subsequent milking. The lambing peak, that overlaps the mating peak in treated animals, showed an advance in lambing of approximately 10 d, compared to controls. Hence, the treated ewes responded earlier to the male effect than controls. The administration of melatonin, presumably, has stimulated the

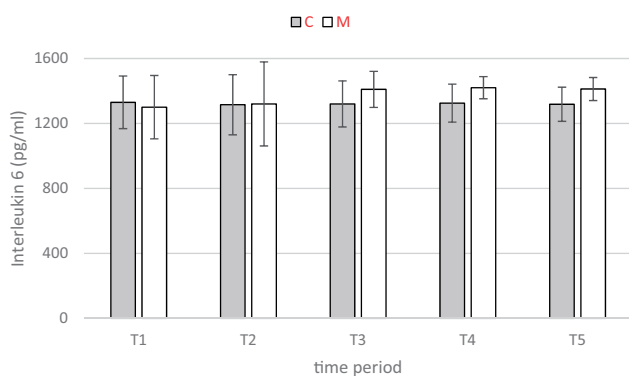


**Figure 1.** Somatic cell score (SCS) trend in M and C groups in the 5 collected samples. M: melatonin treated group; C: control group (without melatonin treatment).  $SCS = \log_2(SCC/100) + 3$ . \*\*\* $p < .01$ .

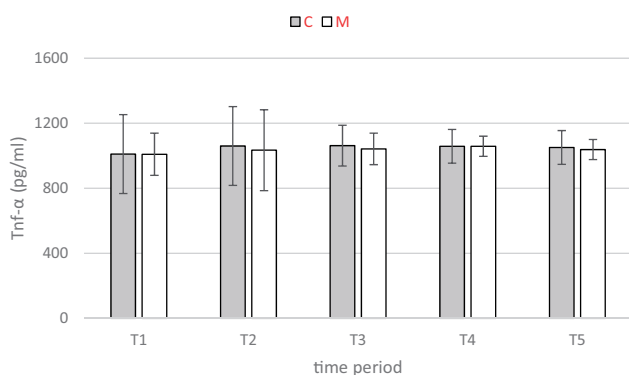


**Figure 2.** Interleukin 2 trend in control (C: untreated) and treated (M: melatonin implanted) ewes in the 5 collected samples.





**Figure 3.** Interleukin 6 trend in control (C: untreated) and treated (M: melatonin implanted) ewes in the 5 collected samples.



**Figure 4.** Tnf- $\alpha$  trend in control (C: untreated) and treated (M: melatonin implanted) ewes in the 5 collected samples.

hypothalamic-pituitary-ovary axis resulting in a shallower anoestrus that led to an earlier reproductive recovery.

In the ovary, the receptors and the synthesis of melatonin have been identified, and this confirms that the action of this indoleamine is important in regulating the ovary activity (Tamura et al. 2009; Casao et al. 2012). Since melatonin receptors were detected in the granulosa cells, that are the only cells in contact with oocytes, it is reasonable to think that this hormone may influence the follicle development (Tamura et al. 2009). Follicles collected by melatonin-treated ewes, in ovum pick-up procedures (OPU), resulted larger and had greater growth in culture than those from untreated sheep (Tsiligianni et al. 2009). Furthermore, melatonin modulates the expression of genes involved in steroidogenesis, which regulate the conversion of progesterone into androgens and the luteinisation of granulosa cells. (Lima et al. 2015; He et al. 2016). The melatonin administration in ewes has shown to increase the progesterone secretion from the corpus luteum (Durotoye et al. 1997; Vázquez et al. 2010). Furthermore, melatonin has antioxidant effects,

reducing free radicals in theca cells, granulosa cells, follicular fluid and corpus luteum, and in this way preserves the health of follicles and the corpus luteum (Reiter et al. 2013; Tamura et al. 2013). Therefore, in this study, all of the actions described above may have affected fertility, also promoting reproductive recovery in melatonin-treated ewes.

Another important result of this study is that melatonin administration did not modify milk yield and quality which remained within the physiological ranges for the Sarda breed (Mura et al. 2013). Abecia et al. (2005) reported that this hormone did not affect the amount of milk produced during lactation in the Assaf and Lacaune breed sheep. Instead, Molik et al. (2011) found that treatment with melatonin implants, or simulation of short photoperiods, caused a decrease in milk levels of solids, protein, fat, lactose and fatty acid content of Polish Longwool sheep milk. In cattle, Dahl et al. (2000) and Ponchon et al. (2017) found that melatonin administration did not change the milk yield and composition. Conversely, Auldust et al. (2007) found that melatonin treatment in cattle decreased milk yield and lactose concentration while increased milk fat, protein and casein content in late lactation. Melatonin administration causes a decrease in prolactin (PRL) secretion, and this reason could lead to the reduction in milk yield (Lacasse et al. 2019). In fact, in cattle the administration of an antiprolactin causes the decline in PRL and a reduction of milk production mainly in late lactation, so favouring the onset of the dry period (Lacasse et al. 2019). Although the role of PRL in lacto-genesis is now recognised, it is still necessary to investigate its exact role in the milk synthesis (Lacasse et al. 2011). In cattle, the antiprolactin administration did not always show the same results, suggesting a different animal response based on the lactation phase (Lacasse et al. 2011). Also, as regards the effect of the melatonin administration on the milk components, the studies conducted have not always provided homogeneous results. It is now known that melatonin modulates lipid metabolism in different species (Le Gouic et al. 1997; Bartness et al. 2002). Furthermore, in a recent study (Wang et al. 2019) the administration of melatonin *in vivo* and *in vitro* in cattle has shown to influence the amount of milk fat produced. However, in our experience as mentioned above, melatonin did not change milk yield and composition, and this may depend on the dosage of the administered melatonin that might be not enough to produce changes in milk synthesis.

In this study, melatonin influenced the number of somatic cells in milk, and this agrees with what found

in cattle, where a reduction in IgG, IgM, lymphocytes and neutrophils was also recorded (Yang et al. 2017). Our data on the reduction in somatic cells after melatonin administration is very important because this research is the first report in a dairy sheep breed. High levels of somatic cells lead to milk depreciation indicating possible sub-clinical mastitis (Albenzio et al. 2012). The sub-clinical mastitis is the main problem of spread and persistent mammary gland infection within the dairy sheep flock. Although the udder and milk seem normal, the mammary gland could be inflamed, infected or both. Sub-clinically infected ewes act as a reservoir for bacteria, resulting in a source of infection for the healthy sheep. The melatonin administration, as shown in our experience, decreased the somatic cells so that we can hypothesise the use of this molecule as a possible means in the fight against sub-clinical mastitis.

In other studies, high melatonin blood level has been associated with the increase in plasma concentration of IL-2 and IL-6 (Garcia-Mauriño et al. 1997; Carrillo-Vico et al. 2003). Differently, high levels of melatonin decrease the expression of  $Tnf-\alpha$  (Di Stefano and Paulesu 1994). In this study, although we found no statistical evidence of the effect of the melatonin treatment on the cytokine's levels, it can be observed an increasing trend for IL-2 and IL-6 compared to controls. This moderate increase in IL-2 and IL-6, and the constancy of  $TNF-\alpha$  levels, in treated animals could be due to the low concentration of melatonin released by the implants. On the other hand, it has been reported that melatonin inhibits  $TNF-\alpha$  secretion in a dose-dependent manner (Espino et al. 2013). In fact, melatonin administration in bovine mammary gland cell culture inhibits the inflammatory process activation (Li et al. 2020) while in goats, the administration of melatonin with food improves cellular-immune function (Singh and Haldar 2017). Therefore, it can be assumed that the modulation of the cell-mediated immune response caused the decrease in somatic cells in the milk of treated ewes.

## Conclusions

Melatonin administration in March confirms the advance of reproductive activity and the improvement of fertility rate in lactating ewes. In addition, the melatonin treatment also resulted in lambing concentration which allows a better rationalisation of the management of lambing, weaning and the subsequent milking. Furthermore, melatonin treatment did not modify neither milk yield nor milk composition. In this study,

for the first time a decrease in milk somatic cells after melatonin administration was reported, indicating a role of melatonin in modulating the immune response in the mammary gland. This important finding encourages further investigations to better understand the role played by melatonin in regulating the immune response in the mammary gland. Thus, the administration of melatonin can be a valid help to keep the mammary gland healthy and avoid the onset of mastitis and so the use of antibiotics against these pathologies.

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