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Subclinical mastitis in Lacaune sheep: Causative agents, impacts on milk production, milk quality , oxidative profiles and treatment efficacy of ceftiofur

Davi Fernando Alba[a], Gilneia da Rosa[a], Dalana Hanauer[b], Thalia Festa Saldanha[c], Carine F. Souza[d], Matheus D. Baldissera[d], Daiane da Silva dos Santos[e], Ana Paula Piovezan[f], Lilian Kolling Girardini[f], Aleksandro Schafer Da Silva[e,*]

[a] Graduate Program in Animal Science, Universidade Do Estado de Santa Catarina (UDESC), Chapecó, Brazil
[b] Food Engineering, Universidade Do Estado de Santa Catarina (UDESC), Pinhalzinho, Brazil
[c] Department of Animal Science, Universidade Federal da Fronteira Sul (UFFS), Realeza, Brazil
[d] Department of Physiology and Pharmacology, Universidade Federal de Santa Maria (UFSM), Brazil
[e] Department of Animal Science, UDESC, Chapecó, Brazil
[f] Department of Animal Science, Universidade Do Oeste de Santa Catarina (Unoesc), Xanxere, Brazil

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ABSTRACT

Mastitis is a major disease affecting dairy sheep. It is caused by microorganisms that generate inflammation of the mammary gland in response to tissue invasion. This syndrome affects the welfare of ewes, as well as the production and quality of the milk, thereby reducing its productive efficiency. Because mastitis causes inflammation process, it also increases the production of free radicals that cause lesions via lipoperoxidation, causing damage to proteins, cells and tissues. One way to minimize the impact of the disease is antimicrobial treatment. Nevertheless, the continuous use of antimicrobials contributes to microbial resistance, in addition to producing residues in the milk and derivatives if not given during the grace period. Therefore, the objective of this study was to evaluate the consequences of subclinical mastitis on ewe health, milk production, milk composition and quality. We also evaluated the susceptibility of the bacteria *in vitro* using disk diffusion antibiograms. Finally, we performed two-way testing of efficacy of treatment in Lacaune ewes using the same agents. In the first stage of the study, 30 lactating ewes (± 90 days) were used, 10 of which were negative on the CMT (California Mastitis Test) used as control group (CG) and 20 sheep with subclinical mastitis diagnosed by CMT (MG). Samples were collected and several analyses were performed on the milk and blood. We found that ewes in the MG had higher lipid peroxidation in serum and milk, as well as lower production, with reduction of the total dry extract in milk. There were 15 isolates of *Staphylococcus hyicus*, four isolates of each *S. epidermidis* and *S. intermedius,* and two isolates of *Corynebacterium* spp. The primary hematological result was leukocytosis in ewes with mastitis. Based on the antibiogram, we chose ceftiofur for *in vivo* tests. In this stage, we divided the sheep with subclinical mastitis into two subgroups of 10 ewes each, to receive drug by two routes: intramuscular (IM) and intramammary (IMM). In the IMM group, of the 10 CMT-positive ewes at the beginning of the experiment, seven were already negative by the racket test 120 h after the last application (70% efficacy). In the IM group, of the 10 positive ewes, only four were negative after 120 h of the final application, a low efficacy treatment (40%). We evaluated antimicrobial residues in the milk of treated animals. We found this material within 5 days after treatment in the two forms used; despite the fact that the product's stated withholding period is 3 days. We conclude that ewes with mastitis produce less milk of lower quality. We also conclude that, although ceftiofur is 100% effective *in vitro*, when used in ewes with mastitis, the efficacy did not exceed 70%, and was more efficient when administered via the intramammary route.

* Corresponding author.
E-mail address: aleksandro_ss@yahoo.com.br (A. Schafer Da Silva).

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1. Introduction

According to the 2017 agricultural census, sheep flocks in Brazil included approximately 13,770,344 head, of which more than 20% were found in the southern region [1]. The total includes sheep destined for production of meat, wool and milk. In 2009, the United Nations Food and Agriculture Organization (FAO) observed that sheep's milk was the fourth most-produced type worldwide, accounting for 1.3% of total production [2]. Since then, sheep milk production has expanded, especially in Brazil [3]. Even so, the production and industrial processing of sheep milk remains small-scale in Brazil. Data collected directly from companies and websites suggest national output of 509,000 L per year [4]. Milk yield is high, which is to say that milk solids levels are high, and Lacaune sheep produce more than 15% solids [5]; the same was observed by Blagitz et al. [6] in Santa Inês sheep; Selaive and Osório [7] point out that sheep milk can reach up to 18.5% solids. This property is important in cheese production. Nevertheless, the solids content of the milk may be affected by the feed supplied to the animals and udder health status.

One of the main problems encountered in sheep farming is mastitis, usually caused by microbiological agents that generate severe local inflammation, compromising the mammary gland and significantly reducing milk production [8]. The strong immune response in the affected ewes affects their welfare and increases the cost of production on account of the need for treatment and disposition [9], as well as mortality in more severe cases serious. Ewes with mastitis may have altered milk composition in terms of fat, protein, and somatic cell counts. This is detrimental to industrial processing, altering coagulation characteristics and cheese production [10], as well as sensory characteristics of the product.

Clinical mastitis is easily identified by changes in milk visible in the dark-bottomed mug test. Subclinical mastitis is characterized by decreased production at the level of ownership and productive yield [11]. An easy way to identify subclinical mastitis is measurement of somatic cell counts (SCC), as well as the California Mastitis Test (CMT) [5].

Mastitis has great economic importance in dairy flocks [12–15]. These studies identified several etiologic agents in milk samples, and found that many of the bacteria were resistant to drugs used on farms. Cortinhas et al. [16] used ceftiofur hydrochloride for intramammary treatment of clinical mastitis in cows and cured more than 70%. Similarly, Oliver et al. [17] used intramammary infusions to treat subclinical mastitis in cows, curing 65.8% with 8 days of treatment. Neto et al. [18] used ceftiofur hydrochloride to treat dry cows 30 days before delivery; nevertheless, antimicrobial residues were detected in 10% of the milk of animals during the first postpartum days. Cristina et al. [19] used ceftiofur intramuscularly in cows to evaluate the residue in milk, and found that it remained for at least 12 h after application. Based on these data, we aimed to treat subclinical mastitis in sheep using ceftiofur intramuscularly and intramammary to evaluate the efficacy of the product used by two routes, as well as to measure its excretion in milk.

Although mastitis is a localized pathology, in clinical cases in ewes, it can have up to 40% mortality when left untreated, especially for mastitis caused by *Staphylococcus aureus* [8]. The same author found that inoculation of the pathogens in the mammary gland caused lost production and one ewe died due to the severity of the infection. In addition, the infectious process can produce metabolites from oxidative reactions and inflammation such as free radicals that circulate throughout the ewe's body, as well as depositing into cells and/or organs, thereby causing injury. In these infectious/inflammatory processes, there is a need for greater energy production, because phagocytosis, regulation of cell growth, intercellular signaling and synthesis of biological substances are important for containing the infection [20]. Excess free radicals cause cell damage by lipid peroxidation (LPO), resulting in oxidation of cell membranes, damaging proteins and DNA, and causing changes in cellular and tissue function, ultimately compromising animal health [21].

We designed the present study because of the importance of mastitis for the production of sheep milk, the lack of information regarding this disease in sheep, and because of the need for more effective treatment alternatives. Therefore, the objective of this study was to evaluate the consequences of subclinical mastitis on ewe health, milk production, milk composition and quality. We also evaluated, *in vitro*, the susceptibility of isolated pathogens using disc diffusion antibiograms, and later *in vivo* tests, to test the two-way efficacy of treatment for ewes using the same agent.

2. Materials and methods

The project was approved by the Ethics Committee on the Use of Animals (CEUA) of the State University of Santa Catarina, protocol number 8278020918. The experiment was carried out in a dairy sheep farm located in the municipality of Chapecó-SC. The study was divided into two stages, as described below.

2.1. STAGE I

2.1.1. Animals and installation

The farm where the experiment was carried out generates sheep milk for the production of derivatives, mainly cheeses, in addition to marketing animals for breeding and meat production. On this farm, antimicrobial therapy has not been used in dairy sheep to treat mastitis for at least five years; this is a requirement of the company that processes and industrializes milk. However, the percentage of ewes with mastitis in the flock was high, affecting approximately 20%, leading to a considerable volume of milk being discarded, thereby reducing the productive efficiency of the farm and resulting in premature ewe culling. In the experimental period, the shed contained approximately 120 Lacaune lactating ewes (approximate weight 70 kg), all in a confined system, of which 30 were selected during the lactation period of approximately 90 days. The ewes were divided into two groups: 20 mastitic ewes (MG), diagnosed using the California Mastitis Test (CMT) and 10 CMT mastitis-negative ewes, used as control group (CG). MG ewes had a mastitis history of more than 30 days and were already in a separate bay from the other productive ewes; however, they did not present with signs such as fever, loss of appetite, apathy, dyspnea or difficulty in locomotion. The ewes were housed in a covered shed, separated by group in two 24 m^2 bays, in wood-shaving covered floors and access to water *ad libitum*. The ewes received the same feed, divided twice a day (7:00 a.m. and 5:00 p.m.), concentrate (17% crude protein), corn silage, and hay.

2.1.2. Milk measurement

On day 0 of the experiment, the milking of the animals was mechanized and performed twice (06:00 and 17:00 h). Individual milked volume was measured using a “Milk Meter” (True Test®, Auckland, New Zeland).

2.1.3. Sample collection

For culturing and antimicrobial susceptibility testing, on day 0, a milk sample of each ewe was collected in a sterile bottle, after cleaning the teat (papilla), disinfection with 70% alcohol and discarding the first three strips. Another 40 mL from each ewe were collected using WB HI/Pullout Tru-Test © equipment, which allowed collection of a milk sample from the complete milking of each ewe. Of the 40 mL, 2 mL were stored in microtubes for evaluation of antioxidant and antioxidant status biomarkers.

After morning milking, while the ewes were fasting, we manually collected blood samples from jugular vein using Vacutainer tubes. Approximately 4 mL of blood were placed in tubes containing EDTA (ethylenediamine tetra acetic acid) for erythrogram and leukogram; another 4 mL were placed in tubes without anticoagulant to obtain

serum for biochemical analyzes and levels of oxidants and antioxidants.

2.1.4. Milk analysis

2.1.4.1. Isolation and identification of microorganisms and antimicrobial susceptibility testing. Samples were cultured in blood agar supplemented with 5% defibrinated sheep blood, MacConkey Agar and Sabouraud Agar. The plates were incubated at 37 °C for 24–72 h and the microorganisms were identified according to morphological characteristics as described by the National Mastitis Council [22] and Markey et al. [23].

Sensitivity profiles of the microorganisms were determined using the disc diffusion method in Müller Hinton Agar, as described by Clinical and Laboratory Standards Institute [24]. We tested disks (LABORCLIN®) impregnated with the following antimicrobials: amoxicillin + clavulanic acid (10 μg), ceftiofur (30 μg), cefalexin (30 μg), ciprofloxacin (5 μg), enrofloxacin (5 μg), streptomycin (10 μg), gentamicin (10 μg), marbofloxacin (5 μg), neomycin (30 μg), oxacillin (1 μg), penicillin (10 IU), tetracycline (30 μg) and trimethoprim + sulfamethoxazole (25 μg). Plates were incubated in a bacteriological oven for 18–24 h at 37 °C. Subsequently, the halos were read and the sensitivity profile of the isolates was determined.

2.1.4.2. Centesimal composition and somatic cell count (CCS) in milk. The centesimal composition of fat, protein, lactose and total dry extract was determined using an infrared analyzer (LactoStar Funke Gerber®) and SCC using a digital counter (Ekomilk Scan Somatic Cell Analyzer®), field equipment used for SCC counting in cow's milk.

2.1.4.3. Oxidant/antioxidant status analysis. Glutathione peroxidase (GPx) activity was measured using *tert*-butyl hydroperoxide as the substrate [25]. Enzyme activity was determined by monitoring the disappearance of reduced nicotinamide adenine dinucleotide phosphate (NADPH) at 340 nm in a medium containing potassium phosphate buffer (100 mM) + EDTA (1 mM), pH 7.7. Results were expressed as U GPx/mg protein. Superoxide dismutase activity (SOD) was determined according to the principle of auto-oxidation of pyrogallol, which is inhibited in the presence of SOD. The variation of optical density was determined kinetically for 2 min at 420 nm, at 10-s intervals, according to the methodology described by Beutler [26]. Activity was expressed as U SOD/mg protein.

Levels of lipid peroxidation (LPO) were measured using the methodology proposed by Monserrat et al. [27]. Results were expressed μmol CHP/mL milk. Nitric oxide levels were measured indirectly as nitrite/nitrate (NOx) levels according to the technique described by Tatsch et al. [28], where 50 μL of sample were pipetted into a reaction cuvette with 50 μL VCl_3. Subsequently, 50 μL of Griess reagent was added and incubated at 37 °C. The readout was performed in 96-well microplates using a SpectraMax I3 (Molecular Devices) plate reader, wavelength 550 nm. The results were expressed as μmol/L. The levels of reactive oxygen species (ROS) in plasma were analyzed by the method described by Ali et al. [29]. The volume of 10 μL of serum were incubated with 12 μL of dichlorofluorescein per 1 mm at 37 °C for 1 h in the dark. Fluorescence was determined using 488 nm for excitation and 520 nm for emission and the results are expressed as U DCF/mL.

Non-protein thiol (NPSH) levels were measured using the DTNB (5, 5′-dithiobis (2-nitrobenzoic acid); Sigma) method as described by Sedlak and Lindsay [30]. NPSH content in the samples was measured after deproteinization with trichloroacetic acid (TCA 50%). Absorbance readings (405 nm) were performed using a spectrofluorimeter (Biotek, Synergy HT).

2.1.5. Blood analyses

2.1.5.1. Hemogram. Total erythrocyte and leukocyte counts as well as hemoglobin concentration was performed using a semi-automated cell counter (CELM model CC530). The differential leukocyte count was performed using blood smears stained by the method of Romanowsky [31] and visualized using light microscopy. The hematocrit was obtained using microhematocrit capillary tubes by centrifuging at 11,000 g for 5 min.

2.1.5.2. Serum biochemistry. Tubes without anticoagulant were centrifuged (5100 *g* for 10 min) for serum separation. The supernatants were transferred to Eppendorf tubes stored at −20 °C until analysis. Levels of total proteins (TP), albumin, triglycerides, cholesterol and urea were measured using a semi-automatic analyzer (Bio-2000 BioPlus®) and commercial kits (Analisa®). Globulin levels were calculated as the difference between total protein and albumin.

2.1.5.3. Oxidant and antioxidant status. The method for measuring serum LPO [27], ROS [29], NOx [28] and NPSH [30] levels, as well as the GPx [25] and SOD [26] activities were the same as for milk described in section 2.1.4.3.

2.1.6. Udder conformation

Udders were evaluated according to the recommendations of Feitosa [32], where external inspection was performed. Udders were classified as normal when the two mammary glands were similar and were arranged in the anatomical position expected for the ovine species, and were considered abnormal when there was discrepancy between the glands, lesions or increased volume such that it would hinder milk production and milking. Udders were also evaluated for the presence (positive) or absence (negative) of masses/nodules by palpation after milking.

2.2. STAGE II

2.2.1. Experimental design

After microbial isolation and antimicrobial susceptibility testing in the first stage of this study, the second stage of the project was started. MG ewes were divided into two subgroups of 10 ewes, in order to form two homogenous groups, blocked based on the isolated pathogens. One subgroup (IM) received intramuscular treatment and the other subgroup (IMM) received intramammary treatment. Only one antimicrobial drug available in commercial formulation was chosen for both routes of application. Based on the susceptibility test of step 1 of this experiment, we chose ceftiofur, a 3rd generation cephalosporin, active against gram-positive and gram-negative bacteria, including those producing beta-lactamases, as follows: 5 g of ceftiofur hydrochloride in 100 mL of vehicle) for intramuscular application; and Spectramast®LC (125 mg of ceftiofur hydrochloride in 10 mL of vehicle) for intramammary application.

In intramuscularly treated ewes, each ewe received 1.5 mL of ceftiofur once daily for three consecutive days (24-h interval). The dose was calculated according to the manufacturer's recommendation for the product, which contains 1 mL for each 50 kg of body weight. In ewes with intramammary treatment, the commercially-available product for the treatment of mastitis in dairy cattle was used. Considering the difference between the size of the mammary gland and the milk production, the product was fractionated in four doses of 2.5 mL. The product (2.5 mL) was injected into the interior of each mammary gland through the teat canal, once daily after morning milking for three consecutive days (24-h intervals).

2.2.2. Sample collection

On day 4 (24 h after the last application) and on day 9 (5 days after the last application) individual milk samples were collected from each ewe (including 5 mL off the top of the harvested milk, representing the fat portion, totaling 10 mL), stored in sterile vials and sent for analysis of antimicrobial residues. On day 9, milk and blood samples from the ewes were also collected for analysis of milk composition and hemogram/biochemical variables, respectively.

2.2.3. Treatment efficiency

Efficacy of the treatments was measured according the results of CMT and SCC on day 4 (24 h after the last application) and on day 9 (120 h after the last application) compared to day 0 (before antimicrobial application).

2.2.4. Antimicrobial residue in milk

To verify the presence or absence of antimicrobial residues, we used the commercial kit Eclipse 50 – Cap-Lab®. The test measures inhibition of microbial growth, using a microtiter plate whose wells contain specific culture medium with *Geobacillus stearothermophilus* spores and an acid-base indicator. After the application of 50 µL of milk, the plates were incubated at 65 °C and the spores germinate and multiply by acidifying the medium and resulting in the indicator changing from blue to yellow-green. If the milk sample contains an antimicrobial concentration higher than the detection limit of the test, the growth of the microorganism is inhibited such that there will be no acid production, nor consequent modification of the color of the medium. The threshold for detection of ceftiofur residues in parts per billion (PPB) was 100 µg/mL.

2.2.5. Milk composition and SCC

The percentage of fat, lactose, protein and total solids, as well as SCC was evaluated according to methodology previously described in stage I.

2.2.6. Hemogram and biochemical analysis

The hemogram and serum biochemistry levels were also evaluated according to methodology previously described in step I.

2.3. Statistical analysis

The data were subjected to normality testing (Shapiro-Wilk). Data that did not present normal distribution (LPO and SCC in milk; total leukocytes, lymphocytes, neutrophil, monocyte and eosinophil in blood; and LPO and GPx in serum) were transformed to logarithms to normalize them. Data were subsequently subjected to comparison of means using two-way ANOVA for comparisons between groups and analysis over time. Significance was considered when $P < 0.05$. The statistical analyses were performed using R-language, v.3.1 (R Development Core Team 2012).

3. Results

3.1. Stage I

3.1.1. Isolates and antimicrobials

Of the 30 samples collected (20 ewes from MG and 10 from CG), there was no growth and microbial isolation in five. In the remaining 25 milk samples, there was growth of *Staphylococcus hyicus* (n = 15, corresponding to 60% of the isolated agents), *S. epidermidis* (n = 4, corresponding to 16% of the isolated agents), *S. intermedius* (n = 4, corresponding to 16% of the isolated agents) and *Corynebacterium* spp. (n = 2, corresponding to 8% of the isolated agents) (Table 1).

Most of the isolated microorganisms were sensitive to the 13 antimicrobials tested, as detailed in Table 1. Only three isolates of *S. hyicus* were resistant to oxacillin, one isolate of *S. intermedius* was resistant to streptomycin, marbofloxacin and oxacillin, and an *S. intermedius* isolate was resistant to tetracycline, just as a *S. hyicus* isolate was resistant to enrofloxacin and tetracycline.

3.1.2. Production, composition and quality of milk

Production (liters/ewe/day), protein content, fat and total solids were lower in the MG than in CG, except for lactose levels that were greater in the MG (Table 2). With respect to SCC, levels were significantly greater in the MG group (Table 2).

Reactive oxygen species (ROS) and LPO levels, as well as GPx and SOD activities were also greater in the milk of the MG group. The NOx levels were lower in the MG group. Non-protein thiol (NPSH) levels were not significantly different between groups (Table 2).

3.1.3. Hemogram

Numbers of erythrocytes, hematocrits, and hemoglobin levels did not significantly differ between groups ($P > 0.05$), whereas total leukocyte values were significantly higher in the MG group as a consequence of a significant increase in neutrophils and lymphocytes ($P < 0.05$). The numbers of monocytes and eosinophils were similar in both groups ($P > 0.05$) (Table 3).

3.1.4. Serum biochemistries and oxidative profile

Urea levels were greater in the MG group than in the CG group ($P < 0.05$). The other biochemical variables (glucose, cholesterol, triglycerides, total protein and albumin) did not significantly differ between groups. Levels of LPO, NOx, and NPSH as well as SOD activity were greater in the MG than in the CG. Levels of ROS and GPx activity were not significantly different between groups ($P > 0.05$).

3.1.5. Udder conformation

Among the 20 ewes in the MG group, 10 (50%) had some alteration of the udder conformation (Fig. 1). Of the 10 ewes in the CG group, only one (10%) had conformational alterations. With respect do masses/nodules, of the 20 ewes in the MG group, 7 (35%) had alterations. Of the 10 ewes in the CG group, one (10%) had nodules.

3.2. Stage II

3.2.1. Efficacy of treatment and clinical evolution

Treatment efficacy is displayed in Table 4. At the beginning of the experiment, 10 ewes in the IM group were positive by the CMT. On day 4 (24 h after the final application) three ewes were had negative CMTs. On day 9, (5 days after the final application), four ewes were negative, with a low efficacy (40%). In the IMM group, of the 10 ewes that were positive on CMT on day 1, on day 4 (24 h after the final application), five ewes were negative on CMT. On day 9 after application, seven ewes were negative, giving an efficacy of 70% for the treatment (see Table 5).

3.2.2. Residual antimicrobial in milk

The results of residual antimicrobial (ceftiofur) in milk are displayed in Table 4. In the IM group, residues were detected in two samples 24 h after the last application and in two samples 120 h after last application. For the IMM treatment, 24 h after the last application residue was detected in all samples, 120 h after the last application, only one sample had residue.

3.2.3. Milk quality and composition

Milk composition did not significantly differ in terms of percentage of protein and fat between the IM and IMM and CG groups. Lactose was higher in the treated groups (IM and IMM) than in the control group (Table 5). SCCs were similar in the treated groups (IM and IMM), but were significantly higher than those of the CG group (Table 5).

3.2.4. Hemogram and serum biochemistries

Blood count variables did not significantly differ between the treated groups (IM and IMM) and GC, except for the number of neutrophils, which was higher in both treated groups (Table 6). There were no significant differences in terms of biochemical variables (glucose, cholesterol, triglycerides, total protein and albumin) between groups Table 6.

4. Discussion

The most prevalent species of microorganism in this study were

Table 1
STAGE I: microorganisms isolated in the 30 dairy sheep; and results of sensitivity testing (S: sensitive; R: resistant) for various antibiotics: AM (amoxicillin + clavulanic acid - 10 μg), CE (ceftiofur - 30 μg), CA (cefalexin - 30 μg), CI (ciprofloxacin - 5 μg), EN (enrofloxacin - 5 μg), ST (streptomycin - 10 μg), GE (gentamicin −10 μg), MA (marbofloxacin - 5 μg), NE (neomycin - 30 μg), OX (oxacillin - 1 μg), PE (penicillin – 10 IU), TE (tetracycline - 30 μg), TR (trimethoprim + sulfamethoxazole - 25 μg).

Sheep[1]	Isolated agent	Antimicrobial												
		AM	CE	CA	CI	EN	ST	GE	MA	NE	OX	PE	TE	TR
M1	*Staphylococcus hyicus*	S	S	S	S	S	S	S	S	S	**R**	S	S	S
M2	*Staphylococcus epidermidis*	S	S	S	S	S	S	S	S	S	S	S	S	S
M3	*Staphylococcus intermedius*	S	S	S	S	S	S	S	S	S	S	S	S	S
M4[3]	*Corynebacterium* sp.	–	–	–	–	–	–	–	–	–	–	–	–	–
M5	*Staphylococcus intermedius*	S	S	S	S	S	S	S	S	S	S	S	S	S
M6	*Staphylococcus hyicus*	S	S	S	S	S	S	S	S	S	S	S	S	S
M7	*Staphylococcus hyicus*	S	S	S	S	S	S	S	S	S	**R**	S	S	S
M8	*Staphylococcus epidermidis*	S	S	S	S	S	S	S	S	S	S	S	S	S
M9	*Staphylococcus hyicus*	S	S	S	S	S	S	S	S	S	S	S	S	S
M10	*Staphylococcus intermedius*	S	S	S	S	**R**	S	S	**R**	S	**I**	S	S	S
M11	*Staphylococcus intermedius*	S	S	S	S	S	S	S	S	S	S	S	**R**	S
M12[2]	—	–	–	–	–	–	–	–	–	–	–	–	–	–
M13	*Staphylococcus hyicus*	S	S	S	S	S	S	S	S	S	S	S	S	S
M14	*Staphylococcus hyicus*	S	S	S	S	S	S	S	S	S	S	S	S	S
M15	*Staphylococcus hyicus*	S	S	S	S	S	S	S	S	S	S	S	S	S
M16	*Staphylococcus hyicus*	S	S	S	S	S	S	S	S	S	S	S	S	S
M17	*Staphylococcus hyicus*	S	S	S	S	S	**R**	S	S	S	S	S	**R**	S
M18	*Staphylococcus hyicus*	S	S	S	S	S	S	S	S	S	S	S	S	S
M19	*Staphylococcus epidermidis*	S	S	S	S	S	S	S	S	S	S	S	S	S
M20	*Staphylococcus hyicus*	S	S	S	S	S	S	S	S	S	S	S	S	S
C1	*Staphylococcus hyicus*	S	S	S	S	S	S	S	S	S	S	S	S	S
C2[2]	—	–	–	–	–	–	–	–	–	–	–	–	–	–
C3	*Staphylococcus hyicus*	S	S	S	S	S	S	S	S	S	S	S		S
C4[3]	*Corynebacterium sp.*	–	–	–	–	–	–	–	–	–	–	–	–	–
C5	*Staphylococcus hyicus*	S	S	S	S	S	S	S	S	S	**R**	S	S	S
C6	*Staphylococcus hyicus*	S	S	S	S	S	S	S	S	S	S	S	S	S
C7[2]	—	–	–	–	–	–	–	–	–	–	–	–	–	
C8[2]	—	–	–	–	–	–	–	–	–	–	–	–	–	–
C9	*Staphylococcus epidermidis*	S	S	S	S	S	S	S	S	S	S	S	S	S
C10[2]	–	–	–	–	–	–	–	–	–	–	–	–	–	

Note 1: Animals M1 to M20 belong to the group with mastitis and C1 to C10 to the control group.
Note 2: No etiological agent was isolated (—), resistance test not performed (−).
Note 3: No antibiogram was performed for *Corynebacterium*, because the technique used is only for fast growing bacteria, as *Corynebacterium* has slow growth the result would not be reliable.

Table 2
STAGE I - production, composition and milk quality of sheep diagnosed with subclinical mastitis compared to control (negative in the CMT test).

Variable	Mastitis group (MG)	Control group (CG)	P-values
Production (L)	0.51 (0.2)	0.85 (0.2)	**0.001***
Protein (%)	2.64 (0.9)	3.95 (0.4)	**0.001***
Fat (%)	4.30 (0.9)	5.93 (0.9)	**0.001***
Lactose (%)	4.93 (0.7)	3.90 (0.30)	**0.046***
Total solids (%)	11.91 (0.8)	13.68 (1.0)	**0.008***
CCS (x10^3 mL)	3059 (1704)	503.1 (456)	**0.014***
Oxidant/antioxidant status			
ROS (U/DCF mg protein)	0.27 (0.08)	0.20 (0.03)	**0.001***
LPO (μmol CHP/mL milk)	164.3 (96.2)	42.2 (29.1)	**0.001***
NOx (μmol/L)	65.4 (10.5)	81.2 (11.0)	**0.023***
NPSH (SH/g of tissue)	0.80 (0.14)	0.81 (0.12)	**0.956**
SOD (U SOD/mg protein)	1.12 (0.41)	0.62 (0.32)	**0.050***
GPx (U GPx/mg protein)	14.2 (5.4)	9.85 (2.3)	**0.047***

*P < 0.05 shows significant difference between groups.

Staphylococcus spp., particularly *S. intermedius*, *S. hyicus* and *S. epidermidis*, corresponding to 92% of the bacterial growth. These species have been reported in the literature as the most prevalent and of greatest importance in the etiology of subclinical mastitis in ruminants ([33–35]). Drescher et al. [36] also observed that *Staphylococcus* spp. were commonly involved in cases of subclinical mastitis in sheep in the west of Santa Catarina, as observed in the present study.

Among the limitations of the study is that there is a paucity of products that treat mastitis in sheep, especially for intramammary application; instead, we needed to adapt treatments used in cattle that may have different levels of efficacy when the dose is extrapolated one species to another. The time of application of the antimicrobial was determined according to the recommendations for cattle, where the product (IMM) should be applied every 24 h and can be used in treatments of 2–8 days, while the injectable product (IM) is recommended for use every 24 h for three days. To standardize the application during the study, we used both products for three days. Another limitation was the quantification of SCC with field equipment made to measure cells in cow's milk; This is because equipment for sheep milk analysis is one or the other; furthermore, reference values have not yet been officially established in Brazil. One other important additional limitation is the lack of established antimicrobial resistance breakpoints for ewe mastitis pathogens.

Among the milk samples used for microbial isolation, bacterial growth was observed in six samples from the control group (60%), and the isolated microorganisms were the same ones that were present in milk samples from ewes with subclinical mastitis measured by the CMT test. According to Kulkarni and Kaliwal [37], these agents are part of the normal microbiota of the skin and udder. Several species of *Staphylococcus* are commonly found in the tear ducts and on the skin of domestic ruminants, whereupon they are introduced to the mammary gland via the act of suction performed by calves or lambs, without infection of the mammary parenchyma. It is important to emphasize that *Staphylococcus* spp. has opportunistic behavior in mammary gland infections, since mastitis caused by commensal organisms occurs when the immunity of the host is compromised or when hygienic sanitary

Table 3
STAGE I - Hemogram, serum biochemistry and status of antioxidants and oxidants in serum of sheep diagnosed with subclinical mastitis compared to control (negative California Mastitis Test).

Variable	Mastitis group (MG)	Control group (CG)	P-values
Hemogram			
Erythrocytes (x10^6 µL)	7.25 (1.53)	6.22 (1.68)	**0.142**
Hematocrit (%)	35.5 (3.78)	33.10 (5.8)	**0.587**
Hemoglobin (mg/dL)	10.7 (1.78)	10.0 (1.20)	**0.624**
Leukocytes (x10^3 µL)	17.1 (5.8)	9.58 (1.9)	**0.001***
Lymphocytes (x10^3 µL)	6.36 (2,5)	3.38 (1,6)	**0.001***
Neutrophils (x10^3 µL)	10.02 (4.3)	5.68 (2.2)	**0.001***
Monocytes (x10^3 µL)	0.72 (0.5)	0.50 (0.27)	**0.534**
Eosinophils (x10^3 µL)	0.03 (0.1)	0.0 (0.0)	**0.667**
Serum biochemistries			
Glucose (mg/dL)	62.0 (7.2)	71.8 (9.4)	**0.207**
Cholesterol (mg/dL)	59.0 (11.3)	68.9 (7.4)	**0.159**
Triglycerides (mg/dL)	21.1 (5.4)	23.0 (4.9)	**0.847**
Total protein (g/dL)	8.25 (1.2)	8.0 (0.87)	**0.924**
Albumin (g/dL)	2.92 (0.4)	2.90 (0.40)	**0.947**
Globulin (g/dL)	5.35 (1.23)	5.12 (0.88)	**0.854**
Urea (mg/dL)	45.6 (8.3)	62.3 (11.3)	**0.001***
Oxidant/antioxidant Status			
ROS (U/DCF mg protein)	1.71 (0.35)	1.44 (0.24)	**0.081**
LPO (µmol CHP/mL serum)	226.2 (134)	52.2 (25.5)	**0.001***
NOx (µmol/L)	156.8 (17.0)	129.2 (19.0)	**0.047***
NPSH (SH/g of tissue)	1.51 (0.22)	1.21 (0.10)	**0.034***
SOD (U SOD/mg protein)	0.89 (0.35)	0.41 (0.23)	**0.050***
GPx (U GPx/mg protein)	7.60 (4.2)	7.2 (5.2)	**0.795**

*P < 0.05 shown significant difference between groups.

conditions are not favorable.

It is also worth mentioning the isolation of *Corynebacterium* spp. in two samples. This microorganism is found in soil and water, and it acts as a secondary pathogen in subclinical mastitis, suggesting transmission at the time of milking, both from the hands of the milker, or from equipment and utensils that have not been not properly disinfected [38]. Mastitis related to this bacterial genus has also been attributed to excessive sucking by the lamb, or by the contact of the skin of the teat with contaminated pastures and beds post-milking [39].

Subclinical mastitis caused by *Corynebacterium* spp. occur rarely and are less worrying because they generally cause a considerable increase in SCCs, facilitating early diagnosis, as does infection by *Staphylococcus* spp. that causes significant losses in milk production by permanent destruction of cells of the glandular epithelium. Consequently, the injured tissue is replaced by fibrous tissue that acts as a form of protection against the invading organism, causing reduction in phagocytosis by neutrophils and the spread of infection via the bloodstream [40].

Corroborating the results of the present study, Bolsanello et al. [39], in a study on the etiology of mastitis in Bergamacia sheep, reported that 61.1% of the isolates were *Staphylococcus* spp. and only 11.2% were *Corynebacterium bovis*. Zafalon et al. [41] isolate *Corynebacterium* spp. in only 0.2%. Nevertheless, the literature considers this agent to be the one with the highest prevalence in the southeast and center-west regions, according to a national data survey performed by Acosta et al. [42]. Unlike *Staphylococcus aureus* infections, other staphylococcal infections, such as those found in this study, are more easily treated and eliminated. Nevertheless, knowledge of the antimicrobial susceptibility profile of the causative agents of this infection is important for treatment success [42].

In this study, the sensitivity profile of the isolated microorganisms did not show great variation. Most of the isolated agents were sensitive to all antimicrobials, a fact explained by the non-use of antibiotics on the farm where the study was carried out for at least five years. Nevertheless, some isolates were resistant to oxacillin, streptomycin, marbofloxacin, tetracycline and enrofloxacin. According to the literature, oxacillin-resistant microorganisms are more resistant to other antimicrobials, especially penicillin, cefepime and gentamicin, compared to other oxacillin-sensitive microorganisms [38]. It is important to perform antibiograms prior to the treatment of animals to increase efficacy and to prevent the spread of bacterial resistance; nevertheless, *in vitro* results are not always reproduced *in vivo*, as occurred in the second stage of this study. Acosta et al. [42] found that the main antimicrobial drugs with resistance problems were penicillin (80%), ampicillin (67%), amoxicillin (67.4%) and neomycin (80%) when tested against microorganisms that cause mastitis in ruminants. In a study carried out in the west of Santa Catarina by Drescher et al. [36] there was a great variation in susceptibility of microorganisms isolated from sheep milk with subclinical mastitis, where novobiocin had the lowest percentage of sensitivity (10.46%), followed by erythromycin (16.29%), lincomycin (17.43%) and amoxicillin (19.77%). However, there is a great diversity of agents involved in cases of subclinical mastitis in sheep, as well as different levels of resistance between regions or even properties, as can be observed by correlating the results of the study with those already mentioned in the literature.

In Brazil today, commercial treatment of mastitis in sheep is usually the systemic route, because it is rare for companies to produce tubes specific for sheep on account of low demand. Santana et al. [43] noted that various antimicrobials have been recommended for intramammary mastitis therapy in sheep; however, these recommendations were based on cattle. The great diversity of organisms responsible for subclinical mastitis and the increasing resistance of these isolates to conventional

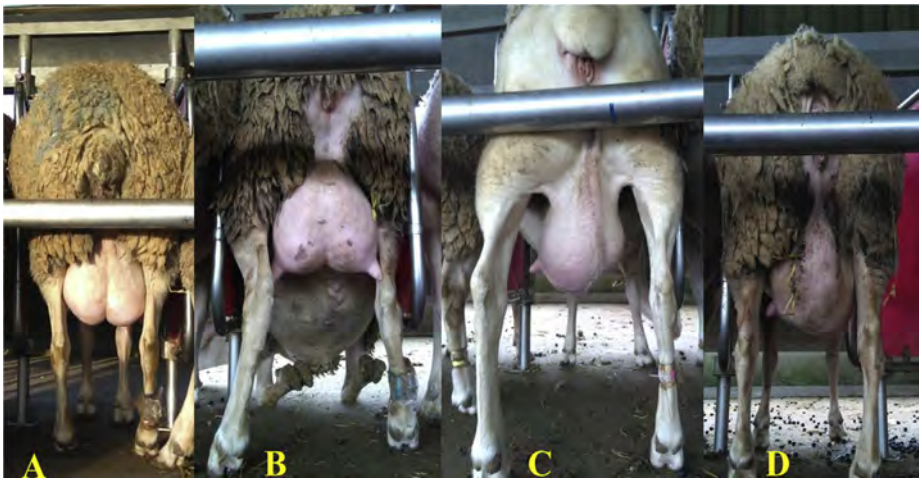

Fig. 1. STAGE I - Udder conformation classification of Lacaune sheep diagnosed with subclinical mastitis compared with control (California Mastitis Test-negative). Udders with normal morphology (A and B) and abnormal morphology (C and D).

Table 4
STAGE I - Results (PO: positive; NE: negative) of the California Mastitis Test (CMT) after intramuscular treatment (IM) or intramammary treatment (IMM) in sheep compared to the control (CON), as a result of the presence (PR) or absence (AU) of antimicrobial residues in sheep milk 24 and 120 h after final application.

Animal/group	Organism	Racket test (CMT)		Residual antibiotic in milk	
IM group		Day 4 (24 h post-last dose)	Day 9 (5 days post-last dose)	Day 4 (24 h post-last does)	Day 9 (5 days post-last dose)
1	*Staphylococcus hyicus*	PO	NE	PR	PR
2	*Staphylococcus epidermidis*	NE	PO	PR	PR
3	*Staphylococcus intermedius*	PO	NE	AU	AU
4	*Corynebacterium sp.*	NE	NE	AU	AU
5	*Staphylococcus intermedius*	PO	PO	AU	AU
6	*Staphylococcus hyicus*	PO	PO	AU	AU
7	*Staphylococcus hyicus*	PO	PO	AU	AU
8	*Staphylococcus epidermidis*	PO	PO	AU	AU
9	*Staphylococcus hyicus*	NE	NE	PR	PR
10	*Staphylococcus hyicus*	PO	PO	AU	AU
Efficacy		**30% (3/10)**	**40% (4/10)**	-	-
IMM group					
1	*Staphylococcus intermedius*	NE	PO	PR	AU
2	*Staphylococcus intermedius*	PO	PO	PR	AU
3	—	PO	NE	PR	AU
4	*Staphylococcus hyicus*	NE	NE	PR	AU
5	*Staphylococcus hyicus*	PO	PO	PR	PR
6	*Staphylococcus hyicus*	NE	NE	PR	AU
7	*Staphylococcus hyicus*	PO	NE	PR	AU
8	*Staphylococcus hyicus*	NE	NE	PR	AU
9	*Staphylococcus hyicus*	NE	NE	PR	AU
10	*Staphylococcus epidermidis*	PO	NE	PR	AU
Efficacy		**50% (5/10)**	**70% (7/10)**	-	-
CON					
1	*Staphylococcus hyicus*	NE	NE	AU	AU
2	–	NE	NE	AU	AU
3	*Staphylococcus hyicus*	NE	NE	AU	AU
4	*Corynebacterium sp.*	NE	NE	AU	AU
5	*Staphylococcus hyicus*	NE	NE	AU	AU
6	*Staphylococcus hyicus*	NE	NE	AU	AU
7	–	NE	NE	AU	AU
8	–	NE	NE	AU	AU
9	*Staphylococcus epidermidis*	NE	NE	AU	AU
10	–	NE	NE	AU	AU

Note: Etiologic agent not isolated (—).

Table 5
STAGE II - Milk composition in the sheep of the three experimental groups (intramuscular –IM; intramammary – IMM; control group – CON) on the 9th day of experiment, i.e., 9 days after starting treatment with chemotherapeutic agents.

Variable	IM	IMM	CON	P-values
Protein (%)	3.81 (0.3)	3.55 (0.6)	3.97 (0.20)	**0.757**
Fat (%)	5.65 (1.2)	6.87 (1.6)	4.90 (1.5)	**0.052**
Lactose (%)	5.63 (0.5)[a]	5.24 (0.9)[a]	3.85 (0.2)[b]	**0.001***
Total solids (%)	14.79 (1.3)[a]	16.0 (1.9)[a]	12.72 (1.0)[b]	**0.001***
CCS (x10^3)	2503 (1463)[a]	1729 (1154)[a]	229.3 (155.2)[b]	**0.001***

P ≤ 0.05 shows difference between groups, identified by different letters (a, b) on the same line.

Table 6
STAGE II - Hemogram and serum biochemistry in the sheep of the three experimental groups (intramuscular –IM; intramammary – IMM; control group – CON) on the 9th day of the experiment, i.e., 9 days after starting chemotherapy.

Variable	IM	IMM	CON	P-values
Hemogram				
Erythrocytes (x10^6 μL)	9.74 (1.4)	10.0 (1,2)	8.45 (1.2)	**0.214**
Hematocrit (%)	32.4 (3.1)	34.4 (4.8)	31.8 (3.2)	**0.567**
Hemoglobin (mg/dL)	9.68 (0.9)	10.3 (1.5)	9.91 (0.9)	**0.814**
Leukocytes (x10^3 μL)	18.83 (9.1)	23.14 (13.0)	9.04 (6.3)	**0.093**
Lymphocytes (x10^3 μL)	8.89 (6.6)	8.40 (5.5)	4.48 (4.0)	**0.310**
Neutrophils (x10^3 μL)	9.30 (3.7)[a]	13.8 (8.7)[a]	3.89 (2.6)[b]	**0.002***
Monocytes (x10^3 μL)	0.63 (0.6)	0.04 (0.1)	0.29 (0.3)	**0.064**
Eosinophils (x10^3 μL)	0.01 (0.09)	0.04 (0.1)	0.02 (0.07)	**0.798**
Serum biochemistries				
Glucose (mg/dL)	77.2 (16)	72.9 (14)	70.2 (8.2)	**0.745**
Cholesterol (mg/dL)	64.1 (24)	69.5 (19)	74.2 (11)	**0.423**
Triglycerides (mg/dL)	25.5 (12)	27.5 (7.8)	24.7 (8.8)	**0.589**
Total protein (g/dL)	8.15 (1.32)	8.73 (0.85)	7.68 (0.71)	**0.235**
Albumin (g/dL)	3.05 (0.42)	3.01 (0.44)	3.16 (0.47)	**0.806**
Globulin (g/dL)	5.10 (1.01)	5.72 (0.94)	4.52 (0.73)	**0.114**
Urea (mg/dL)	47.9 (8.9)	48.5 (14.1)	43.8 (7.6)	**0.501**

*P < 0.05 shows difference between groups, identified by different letters (a, b) on the same line.

antimicrobials highlight the need to organize therapeutic protocols with support in microbial sensitivity tests, because only some drugs are licensed and are available for use in small ruminants. Thus, sheep farmers use anti-mastitic agents and other drugs for other species, creating a high risk to the safety of these ewes, as well as to their effectiveness, because little is known about the great majority of the drugs in dairy sheep.

Ceftiofur was 100% effective in *in vitro* tests and, because it is found on the local market in two different routes of administration, it was chosen for this study. The efficacies of IM and IMM treatment were different, being greater for IMM (70%). This efficacy is nevertheless considered low; therefore, further studies should be developed to define the best dose as well as to produce commercially suitable delivery devices for ovine species. We recorded low efficacy for the IM route, one of the main routes used to control mastitis in sheep, probably to producers having difficulty finding proper tubes for sheep in the Brazilian market. The use of ceftiofur hydrochloride for the treatment of clinical mastitis in cows had an efficacy of more than 70% [16]. In a study with cows with subclinical mastitis, 65.8% were cured with intramammary

application over 8 continuous days [17]; these efficacy values were similar to those in the present study, with intramammary ceftiofur over 3 consecutive days.

According to the manufacturer, the commercial product based on ceftiofur when applied by IMM has a withholding period of 72 h for cows; that is, the minimum period that the milk of the animal should not be used. This same product was used for sheep and found that even after 120 h of the last application, one animal (10%) tested positive for antibiotic residue in milk. This highlights the fact that commercial products produced for cattle and other species have different withholding periods. In the technical recommendations of the commercial product based on ceftiofur used in the IM application, there is no mention of withholding period for the use of cow's milk for human consumption. Nevertheless, in sheep's milk, ceftiofur residues were detected in 3 milk samples 120 h after application.

The presence of antimicrobial residues in the milk represents a risk to the consumer, and is a serious problem for economic and public health [44]. The economic consequences relate to undesirable effects in the manufacture of dairy products [45]. Antimicrobial residues can influence the quality of the derivatives by inhibiting the fermentation of lactic acid bacteria in the production of yogurt, cheese and butter, causing serious damage to the dairy industry. Public health problems have been pointed out since the 1950s [46], when antimicrobial residues such as penicillin in milk sensitized non-allergic individuals, and caused allergic reactions in previously sensitized patients. In the 1990s, Costa [47] and Albuquerque et al. [48] reported the selection of resistant bacterial strains in the environment caused by the exaggerated use of antimicrobials in animals and in the human gastrointestinal tract after ingestion of antimicrobial residues present in food; therefore, countries have created policies to reduce food contamination by antimicrobials, including banning their use in animal feed. Brazil published decree 171 in December 2018 [49].

During the invasion of the mammary gland by the microorganisms, the number of the defense cells, primarily neutrophils, is increased in order to combat the infectious process, resulting in reduced production and changes in milk composition, concomitant with increased somatic cell counts [50]. This process was observed in the MG, where the ratio of SCC and neutrophils was higher than that of the CG, suggesting that infection with the isolated agents caused severe damage to the glandular tissue, even in subclinical infection. Coelho et al. [51] demonstrated that milk with high somatic cell counts results in alterations of the derivatives, where the cheese presented lower protein content, higher humidity and lower industrial yield, reducing the shelf life of the product. Rovai et al. [10] found that milk from sheep with mastitis also have higher SCC, lower coagulation capacity, and higher serum and protein losses during the coagulation process, where the final product (cheese) was softer and more elastic, probably due to the higher water content. In order to avoid further deterioration of the milk, Rovai et al. [10] recommended that milk from mastitis be processed faster, because the degradation of this milk is greater than the milk of healthy ewes.

Serum NOx levels were higher in sheep with mastitis, whereas in milk, these levels were lower. Although these ewes had chronic mastitis, NOx levels remained high in the blood, probably as a vigilant defense mechanism, keeping bacterial growth controlled, even though they caused only subclinical mastitis. The increase in nitric oxide is normal in acute conditions because of its role in the inflammatory processes, and its metabolism increases considerably in the context of greater flow of epithelial cells and macrophages in processes such as those. Jungi [52] points out that nitric oxide has antimicrobial action, since activated macrophages synthesize NO. We do not have an explanation for the observed difference in NOx levels in whey and milk; however, they may be due to low levels of nitrite/nitrate excretion in milk.

As previously mentioned, the number of neutrophils was higher in ewes with mastitis, a characteristic of the bacterial agents involved in the infection. Pinheiro-Junior et al. [53] in cases of acute mastitis caused by *Corynebacterium pseudotuberculosis*, reported a large release of common adrenocortical substances at the beginning of the infection, reflecting an increase of total leukocytes in infected animals; however, with the chronicity of the disease, the values tend to remain within physiological ranges for the cows [54], possibly explaining why difference was observed between the other variables in the current study.

Increased lipid peroxidation in serum and milk of GM ewes may reflect the inflammatory process caused by mastitis. Ebrahimi et al. [50], reported an increase of neutrophils in order to combat the infectious process. This increase in circulating leukocytes results in higher production of oxidants such as ROS, which also act to combat invasive microorganisms [55]. However, the exacerbated increase in the production of reactive oxygen species can cause lipoperoxidation of cell membranes, consequently causing cellular and tissue damage. It is important to note that high concentrations of ROS in many cells induce the expression of genes whose products exhibit antioxidant activity [56]. In healthy animals, there is constant redox signaling between ROS production and the elimination capacity of ROS. If the initial ROS increase is relatively small, the antioxidant response may be sufficient to compensate for the increase of ROS and to redefine the original long-term equilibrium. These mechanisms tend to maintain a stable state called redox homeostasis [56]. In cases of imbalance, e.g., bacterial infections, there is greater production of oxidizing compounds (excessive generation of free radicals or to the detriment of their removal speed) and oxidative stress is established that inhibits tissue remodeling and healing of lesions caused by infectious agents [57].

The antioxidant enzymes SOD and GPx responded to the increase of oxidative reactions; in other words, their activities increased in MG ewes. This may be interpreted as a positive reaction of the organism to the inflammatory process, preventing the establishment of a framework of oxidative stress, because the antioxidant systems were activated, protecting cells against ROS [58]. Atakisi et al. [59] investigated total oxidation and antioxidant capacity in the milk of cows with subclinical mastitis, and found that concentrations of cellular oxidation biomarkers were higher in cows with the disease. These authors suggest that this alteration is a form of protection and reaction of the animal. Ellah [60] notes that mastitis affects the milk production of animals and decreases antioxidant defenses, noting that supplementation with vitamin E, C, beta-carotene and minerals helps in the recovery of animals. A recent study has shown that adding grape flour to sheep diets increases antioxidant levels in milk [61], so these natural additives may be an option in sheep farming.

5. Conclusion

Subclinical mastitis negatively affected udder conformation, production, composition and milk quality in Lacaune sheep. Although the sheep were clinically healthy, subclinical mastitis impaired their health, and they endured inflammatory processes that were intensely activated, reflecting higher energy expenditures as well as greater lipid peroxidation. Bacterial agents were present in the mammary gland of sheep, but did not cause clinical mastitis. The main isolated microbiological agents were those commonly described in ewes with mastitis. Low levels of antimicrobial resistance were detected in this study, possibly as a consequence of the absence of treatment with chemotherapeutic agents in ewes with mastitis. We also concluded that ceftiofur via both routes of administration had low efficacy; however, the intramammary route had 70% grater efficacy than that of the intramuscular route. Antimicrobial residue (ceftiofur) was found in the milk of some sheep within 120 h after application, exceeding the manufacturer's recommended shelf life for cows. This finding suggests that it is important to use commercial products specific for sheep, in light of the fact that production of dairy sheep has increased in Brazil.

Conflicts of interest

The authors declare no conflict of interest.

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