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Microbiological Quality and Somatic Cell Count of Ewe Milk with Special Reference to Staphylococci

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ABSTRACT

A total 1502 useful half udders of 762 Churra ewes from eight herds were aseptically sampled in midlactation to study both the bacteriological isolates and the SCC of milk. Corynebacteria, enterococci, micrococci, staphylococci, and streptococci represented 11.2, 2.9, 1.4, 78.9, and 3.1% of all isolates, respectively. Within staphylococci, novobiocin-sensitive species (71.1%) were much more frequently isolated than novobiocin-resistant ones (7.8%). *Staphylococcus epidermidis* was the most prevalent species (53.2% of the isolates). Log SCC of uninfected half udder milk was 4.86. Isolates of novobiocin-resistant coagulase-negative staphylococci, micrococci, and corynebacteria were associated to low values of log SCC (4.85 to 5.20). In contrast, infection by novobiocin-sensitive coagulase-negative staphylococci, streptococci, and enterococci organisms was related to a sharp inflammatory response with log SCC means between 5.92 and 6.32. The species that showed the highest log SCC were *Pasteurella haemolytica* (7.62), *Streptococcus agalactiae* (7.28), and *Staphylococcus aureus* (6.68). High prevalence of infections by novobiocin-sensitive staphylococci together with high SCC related to such infections show a relevant role of these organisms in ewe mastitis. Consequently, implementation of staphylococcal mastitis control programs would be of great interest in dairy ewe herds to improve microbiological and hygienic quality of milk.
(**Key words:** ewe, mastitis, microbiological quality, somatic cell count)

Abbreviation key: CNS = coagulase-negative staphylococci, **NRS** = novobiocin-resistant staphylococci, **NSS** = novobiocin-sensitive staphylococci.

INTRODUCTION

The prerequisite to producing hygienic milk is udder health. Mastitis, particularly subclinical and chronic, is the most persistent and widely spread group of diseases of importance to milk hygiene in dairy cattle (Heeschen, 1987). Previous studies have confirmed that bacteriological examination of milk and milk SCC are reliable methods for detecting subclinical mastitis in dairy ewes (Marco, 1994; González-Rodríguez, 1995), and an inverse relationship between SCC and milk yield has been proved (Gonzalo et al., 1994). Traditionally, the most common mastitis-causing agents have been classified as minor and major pathogens according to the degree of inflammation they produce in the mammary gland. The most prevalent etiological group is represented by staphylococci and particularly by coagulase-negative staphylococci (**CNS**) (Deinhofer, 1993; González-Rodríguez et al., 1995), considered to be minor pathogens or commensals by many authors (Dohoo and Meek, 1982; Rainard and Poutrel, 1982; Bergonier et al., 1996; Poutrel, 1996). However, there is some evidence in dairy ewes that some CNS species can present high SCC (Marco, 1994; Pengov, 2001) and even cause clinical mastitis (Fthenakis and Jones, 1990) in a way similar to major pathogens. In this respect, sensitivity to novobiocin has been mentioned as a criterion associated with staphylococci pathogenicity (Gutiérrez et al., 1990), although the relationship between degree of sensitivity to this antibiotic and SCC is not known. In addition, a second group of organisms containing classic major pathogens (e.g., streptococci, *Staphylococcus aureus*, coliforms) cause SCC over 600×10^3 cells/ml in cows (Dohoo and Meek, 1982), but nonclinical mastitis prevalence and SCC elicited by these organisms have been less comprehensively studied in dairy ewes (González-Rodríguez et al., 1995). Therefore, better knowledge of pathogens involved in mammary infection and of their relationship with SCC is necessary for an adequate applicability of SCC for diagnosing subclinical mastitis in dairy ewes and to attain ewe milk quality standards demanded by the milk industry.

The objectives of this study were first to examine the etiology of mammary infections in dairy ewes in the Mediterranean production system, and second to study the relationship between type of gland infection and SCC in milk, especially in staphylococci infections.

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MATERIALS AND METHODS

Flocks and Ewes

In a survey study, a total of 1508 samples of half udder milk were collected aseptically in midlactation (75 to 105 d postpartum) from 762 Churra ewes belonging to eight separate flocks in the Castile-León region of Spain. Of the sampled glands, 826 corresponded to four machine-milked flocks and 682 to another four hand-milked ones. All ewes in midlactation were sampled. Midlactation was chosen for a twofold purpose: a) to avoid the lowered responsiveness of mammary gland immunity over the peripartum period (Mallard et al., 1998), and b) to avoid the significant increase of SCC at both weaning (30 d postpartum) and the end of lactation (Gonzalo et al., 1996; Fuertes et al., 1998) as a result of a decrease in milk yield. None of the flocks was included in any mastitis control program and hygienic. No teat dips, antibiotic treatments at drying off, or forestripping before milking were carried out.

Sampling

Before milking, teats were carefully cleaned with cotton wool impregnated with 70% ethanol. After the first streams of milk were discarded, 5 to 10 ml from each half udder were collected into sterile containers. Samples were kept at 4°C until bacteriological analysis, which was carried out immediately on arrival in the laboratory after sampling.

Bacteriology

A 0.02-ml aliquot of each sample (Marco, 1994; Contreras et al., 1997) was spread evenly on 5% sheep blood agar (bioMérieux S.A., Marcy l'Etoile, France) by streaking of the inoculum, beginning at the outside edge of the plate, onto the surface of the medium using calibrated and sterile disposable plastic loops. The plates were incubated aerobically at 37°C and examined after 24 and 48 h. According to Contreras et al. (1997) and Marco (1994), subclinical IMI was defined as growth of five or more identical colonies (≥250 cfu/ml). In the case of *S. aureus*, isolates from one colony per inoculum (≥50 cfu/ml) were considered positive. This aliquot size and definition of IMI allowed us to obtain high sensitivity and specificity values (>96.0%) in the diagnosis of persistent infections from a single milk sample (Contreras et al., 1997). Absence of growth or growth of fewer than five colonies of the same type (<250 cfu/ml) was defined as negative culture. Growth of two different types of colonies with more than five colonies per type was defined as mixed culture. Growth of three or more bacterial types was considered as contaminated culture and eliminated from the study. Finally, clinical culture was that from half udders with clinical mastitis at sampling time.

For each milk sample, isolates were selected from the original sheep blood agar plates according to phenotypical traits of colonies (morphology, color, size, presence, and type of hemolysis). All isolates subcultured from original agar plates were characterized. The methods used to identify the different organisms were those recommended by the National Mastitis Council (1990), with the modifications introduced by Marco (1994) in dairy ewes. Gram staining and the preliminary assays of the catalase for the gram-positive organisms and of the oxidase (Difco Laboratories, Detroit, MI) and fermentation pattern in TSI (Oxoid Ltd., Basingstoke, England) for the gram-negative ones were carried out in all cases.

Micrococcaceae strains (gram-positive, catalase-positive) were differentiated using the lysostaphin resistance test (Schleifer et al., 1981), thus classifying them as micrococci or staphylococci. The species in both groups were identified using ID 32 Staph micromethods (bioMérieux S.A., Marcy l'Étoile, France). Staphylococci were subjected to a disk diffusion susceptibility test, as recommended by Bauer et al. (1966) and NCCLS (1990). Novobiocin disks were purchased commercially (Oxoid Ltd.) and the concentration of novobiocin in the disks was 30 μg. Colonies were inoculated into trypticase soy broth and incubated to attain a turbidity equivalent to, or be adjusted to, the turbidity of a 0.5 McFarland standard. Each isolate was inoculated onto the dried surface of a Mueller-Hinton agar plate (bioMérieux S.A.) by using a swab to streak the inoculum over the entire agar surface. After 16 to 20 h of inoculation at 35°C, the diameters of the zones of complete inhibition of visible bacterial growth around each disk were measured in millimeters. This allowed this group of organisms to be divided into two subgroups: novobiocin-resistant staphylococci (**NRS**), with an inhibition diameter <23 mm and novobiocin-sensitive staphylococci (**NSS**), with an inhibition diameter ≥23 mm. Identification of *S. aureus* was carried out using a rapid agglutination test with blue latex particles coated with porcine fibrinogen and rabbit IgG (Staphytect, Oxoid Ltd.).

Gram-positive, catalase-negative cocci were identified as belonging to the *Streptococcaceae* family and subjected to CAMP test and esculin hydrolysis. CAMP-positive esculin-negative strains were subjected to agglutination of latex particles sensitized by Lancefield group B specific rabbit immunoglobulins (Slidex Strepto B, bioMérieux S.A.); the positive strains were identified as *Streptococcus agalactiae*, while the remaining ones were identified using the API 20 Strep micromethods (bioMérieux).

The gram-positive, pleomorphic and catalase-positive coccobacilli were classified within the *Corynebacterium* genus, whereas catalase-negative ones were identified as *Arcanobacterium pyogenes.* Gram-negative organisms were identified using the PASCO system (Difco Laboratories). Presence of mycoplasma was examined in bulk tank milk samples and those from glands with SCC $\geq 200 \times 10^3$ cells/ml (González-Rodríguez et al., 1995), which did not present any bacterial growth. Mycoplasma analyses were conducted using PPLO medium (agar and broth). Twenty microliters of milk was spread onto the surface of agar plates, and 40 μl was inoculated into a tube containing 2 ml of broth and incubated at 37°C for 48 h in a 5% CO_2 enriched atmosphere. After incubation period, 20 μl was spread onto agar plates and other 20 μl was inoculated into a new broth. Three successive passages from liquid to solid medium, and from liquid to liquid medium, were carried out and all plates were incubated at the same conditions over a period of 10 d and examined daily for the presence of mycoplasmas before considering the culture negative.

SCC

After bacteriological plating, SCC was determined for each milk sample with a Fossomatic 90 (A/S N Foss Electric, Hillerød, Denmark) between 24 and 48 h postcollection using the previously described method (Gonzalo et al., 1993).

Statistical Analysis

Descriptive statistics were carried out to determine prevalences of infection, percentage of isolates and log SCC (±SD) of bacterial genera and species. Student's *t*-test was done to establish differences between NSS and NRS for variables log SCC and diameters to novobiocin. This test was carried out according to the computer program STATISTICA 4.0 (Statsoft, Inc., Tulsa, OK).

RESULTS AND DISCUSSION

Of the total 1508 samples obtained, six corresponded to cases of clinical mastitis (three in machine-milked flocks and three in hand-milked ones), 872 to negative cultures, 615 to positive cultures (576 pure cultures and 39 mixed ones) and 15 to contaminated cultures. Clinical mastitis cases were caused by *S. aureus* (three isolates), *Corynebacterium* spp. (one isolate), a mixed culture made up of *S. aureus* and *Staphylococcus epidermidis* and another by *Enterococcus* spp. and *Micrococcus varians.* The contaminated cultures represented 1% of samples from asymptomatic glands, a value very similar to that obtained in other studies of dairy ewes (Marco, 1994), and were excluded from the study. Fifteen strains of staphylococci did not grow adequately on Mueller-Hinton agar despite repeated attempts, and so it was not possible to obtain reliable inhibition diameters in disk diffusion susceptibility test. Finally, seven milk samples could not be processed for SCC for different reasons such as not enough milk volume or macroscopic milk alteration within 24 to 48 h postcollection.

Global prevalence of subclinical IMI was 41.4%, similar to 39.8% obtained by González-Rodriguez et al. (1995) for the same breed in the same geographical area. Table 1 shows the relative prevalence of each genus, group, and bacterial species for total isolates. Thus the *Staphylococcus* genus was mainly responsible for subclinical infections with a global prevalence of 34.7%. The *Corynebacterium* genus, isolated in 4.9% of half udders, was the second most prevalent one, whereas *Streptococcus* and *Enterococcus* genera had a joint prevalence close to 2.6%. The rest of the genera were isolated in fewer than 1% of the glands. As to species, *S. epidermidis* was by far the most frequently isolated (23.4%), followed by *Staphylococcus xylosus* (3.1%), *Staphylococcus chromogenes* (1,8%), *S. aureus* (1.7%), *Staphylococcus simulans* (1.3%), and *Staphylococcus haemolyticus* (1.1%).

For isolate percentage (Table 1), *Staphylococcus* genus was isolated in 78.9% of positive cultures, with CNS representing 74.8% of isolates, in agreement with values found in dairy breeds by other authors (De la Cruz et al., 1994; Marco, 1994; González-Rodríguez et al., 1995). Within this genus NSS (71.1%) were isolated much more frequently than NRS (7.8%). The most frequently isolated species was *S. epidermidis* (53.2%), in agreement with Marco (1994) in dairy ewes. *Corynebacterium* genus represented 11.2% of isolates, a value similar to 10.1% recorded by Las Heras et al. (1999) and slightly above 6.8% stated by Marco (1994) for dairy sheep. The significant link of this genus to machine milking (24.7%) in comparison with the hand milking (1.8%) could partially explain the discrepancies in the literature about its significance as an etiological agent of subclinical mastitis in dairy ewes (Marco, 1994; Bergonier et al., 1999).

Streptococcaceae family isolates were distributed between *Streptococcus* (3.1%) and *Enterococcus* (2.9%) genera. The global percentage of isolates (6.0%) was similar to 8.2% found in previous studies on the same breed (González-Rodríguez et al., 1995), although lower than that recorded in subclinical mastitis for other dairy (González-Rodríguez et al., 1995) and meat (Maisi et al., 1987; Watkins et al., 1991; Keisler et al., 1992) breeds. *Enterococcus faecalis* was the most frequently

Table 1. Prevalence, isolates, and log SCC (± SD) of each bacterial species and genus found in ewe milk.

Species	N[1]	Prevalence (%)	Isolates (%)	N[2]	Log SCC	SD
Staphylococcus genus	516	34.70	78.90	468	5.90	0.88
Novobiocin-sensitive Staph.	465	31.27	71.10	426	5.99	0.85
S. epidermidis	348	23.40	53.21	327	5.93	0.84
S. chromogenes	27	1.82	4.13	25	6.09	0.76
S. aureus	26	1.75	3.98	21	6.68	0.95
S. simulans	19	1.28	2.91	18	6.35	0.44
S. haemolyticus	15	1.01	2.29	10	5.96	0.88
S. hominis	8	0.54	1.22	7	6.05	0.87
S. caprae	5	0.34	0.76	3	6.43	0.47
S. capitis	2	0.13	0.31	2	6.00	0.90
S. hyicus	1	0.07	0.15	1	5.86	. . .
S. auricularis	1	0.07	0.15	1	5.53	. . .
S. intermedius	1	0.07	0.15	. . .	. . .	. . .
S. spp.	12	0.81	1.83	11	5.66	0.91
Novobiocin-resistant Staph.	51	3.43	7.80	42	4.99	0.55
S. xylosus	46	3.09	7.03	37	4.95	0.57
S. lentus	5	0.34	0.76	5	5.26	0.23
Corynebacterium genus	73	4.91	11.16	64	5.20	0.51
Streptococcus genus	20	1.34	3.06	12	6.32	1.02
Str. agalactiae	4	0.27	0.61	4	7.28	0.57
Str. uberis	4	0.27	0.61	3	5.76	1.21
Str. bovis	3	0.20	0.46	3	6.09	0.32
Str. acidominimus	2	0.13	0.31	. . .	. . .	
Str. spp.	7	0.47	1.07	2	5.60	0.61
Enterococcus genus	19	1.28	2.91	12	5.92	0.96
E. faecalis	7	0.47	1.07	3	6.15	0.38
E. durans	2	0.13	0.31	1	5.24	. . .
E. spp.	10	0.68	1.53	8	5.92	1.12
Micrococcus genus	9	0.61	1.38	7	4.85	0.61
M. luteus	3	0.20	0.46	2	4.65	0.29
M. roseus	3	0.20	0.46	3	4.73	0.57
M. lylae	1	0.07	0.15	1	5.96	. . .
M. spp.	2	0.13	0.31	1	4.49	. . .
Pasteurella genus[3]	2	0.13	0.31	1	7.62	. . .
Arcanobacterium genus[4]	1	0.07	0.15	1	5.46	. . .
Gram-Positive O.	13	0.87	1.99	8	5.30	0.83
Gram-Negative O.	1	0.07	0.15	1	4.68	. . .
Total	654	43.98	100.00	. . .	. . .	. . .
Not infected	872	58.64		868	4.86	0.52

[1]Number of isolates (pure and mixed IMI).

[2]Number of glands with pure IMI.

[3]*Pasteurella haemolytica.*

[4]*Arcanobacterium pyogenes.*

isolated species, followed by *Str. agalactiae* and *Streptococcus uberis.*

Micrococcus genus was only isolated in 1.4% of the positive cultures, which revealed its low etiological significance in ovine mastitis, in agreement with the results of other authors (Marco, 1994).

Pasteurella haemolytica (0.3%) and *A. pyogenes* (0.15%) presented very low isolation frequencies, which was consistent with the very low relative prevalence of these organisms in subclinical processes and with the greater frequency with which they have been described in clinical mastitis (Jones, 1991; Marco, 1994). *Mycoplasma agalactiae* was not isolated either from bulk tank milk or from half udders.

Log SCC for each of the bacterial genera and species found is shown in Table 1. Milk samples from bacteriologically negative half udders showed log SCC of 4.86, in full agreement with other studies of dairy ewes (Marco, 1994; González-Rodríguez et al., 1995). Log SCC in half udders infected by *Staphylococcus* genus was 5.90. NSS species showed a log SCC mean of 5.99, which allows them to be classified as true major pathogens. In contrast, NRS species were related to significantly lower values ($P < 0.001$) of log SCC (4.99) than NSS species. Likewise, infections by *Micrococcus* spp. (4.85) and *Corynebacterium* spp. (5.20) had low log SCC, and so they could be classified as minor pathogens.

Half udders infected by *Streptococcus* and *Enterococcus* genera showed high log SCC (6.32 and 5.92, respectively), *Str. agalactiae* (7.28) standing out as the species that showed the sharpest inflammatory response. The great variety in SCC response shown by the different

species of *Streptococcaceae* family was similar to that recorded by Peris et al. (1997) in Merino ewes (geometric means between 193 and 41,100 $\times$ 10^3 cells/ml).

Finally, *P. haemolytica* and *A. pyogenes,* in the only isolates from these species obtained in pure culture, were related to log SCC of 7.62 and 5.46, respectively, though these SCC values should be interpreted cautiously due to low incidence of both organisms in the sampled population.

Within the different staphylococci species, *S. aureus* showed a sharp mammary inflammation, with a log SCC mean of 6.68. Within CNS species, *Staphylococcus caprae* and *S. simulans* were associated to high log SCC (6.43 and 6.35, respectively). *S. chromogenes* (6.09), *Staphylococcus hominis* (6.05), *Staphylococcus capitis* (6.00), *Staphylococcus haemolyticus* (5.96) and *S. epidermidis* (5.93) showed a less intense inflammatory response, though greater than that found for enterococci (Table 1). Similar SCC (between 930 and 2487 $\times$ 10^3 cells/ml) have also been recently recorded in dairy ewes (Pengov, 2001) for some of these CNS species (*S. epidermidis, S. caprae, Staphylococcus hyicus,* and *S. simulans*), showing that in dairy ewes CNS raise inflammatory indicators in milk as effectively as major pathogens. On the contrary, *Staphylococcus xylosus* and *Staphylococcus lentus* isolates had very low SCC.

These results are in accordance with evidence that some CNS species belonging to the novobiocin-sensitive group, such as *S. epidermidis, S. simulans, S. chromogenes, Staphyloccocus warneri,* and *S. haemolyticus* have been described as responsible for clinical udder alterations and clinical mastitis in ewes (Gutiérrez et al., 1990; Deinhofer, 1993; Marco, 1994), whereas *S. xylosus* and the other NRS species are barely described in such processes. In this respect, Fthenakis and Jones (1990) managed to induce clinical mastitis with histopathological alterations of the mammary gland using *S. chromogenes* and *S. simulans* inoculation, whereas *S. xylosus* inoculation only caused a transitory SCC increase. Similarly, high pathogenicity of *S. haemolyticus* has been experimentally verified by Coni et al. (1999).

The study of the agar disk diffusion zone diameters obtained with novobiocin for *Staphylococcus* spp. (Table 2) showed significantly higher diameters ($P < 0.001$) for NSS (35.0 $\pm$ 3.7) than for NRS (18.0 $\pm$ 2.0), which allowed both subgroups of staphylococci to be easily differentiated. However, the reasons why NSS are related to high SCC or to a higher pathogenicity of these organisms are unknown.

As NSS represent 71.1% of isolates (Table 1) in ewe subclinical infections, these results are of great practical interest and justify the inclusion of novobiocin in pharmacological combinations for the control of non-clinical mastitis. Thus, the efficacy of dry therapy including this antibiotic has recently been proved in field studies (Marco, 1994; Tardáguila, 1999).

Table 2. Arithmetic means and standard deviation of the agar disk difussion zone diameters obtained with novobiocin for *Staphylococcus* species.

		Diameter (mm)	
Staphylococcus	N	X	SD
Novobiocin-sensitive *Staphylococci*	411	35,0	3,7
S. epidermidis	318	35,5	3,2
S. chromogenes	23	36,0	2,8
S. aureus	20	31,1	3,7
S. simulans	18	30,9	3,9
S. haemolyticus	10	31,1	4,8
S. hominis	7	35,9	6,1
S. caprae	2	34,5	3,5
S. capitis	1	35,0	—
S. auricularis	1	35,0	—
S. hyicus	1	39,0	—
S. spp	10	32,9	4,0
Novobiocin-resistant *Staphylococci*	42	18,0	2,0
S. xylosus	37	18,0	2,0
S. lentus	5	18,2	1,8

CONCLUSIONS

CNS represents the most prevalent etiological group found in subclinical infections of dairy ewes. However, most species in this group were associated with very high SCC values, similar to those of major pathogens. Consequently, CNS organisms need to be reassessed as major or minor pathogens since grouping of all CNS species as minor pathogens proves inadequate. Novobiocin susceptibility testing allowed CNS to be divided into two clearly differentiated subgroups as regards milk SCC, but further studies will be necessary to consider this test as a reliable basis on which to reclassify staphylococci in dairy ewes. Additionally, evidence of high milk SCC linked to subclinical infections by NSS emphasizes the need to optimize mastitis control programs in flocks in order to improve microbiological and hygienic quality of milk.

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