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REVIEW ARTICLE

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Milk hygiene in small ruminants: A review

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Abstract

Somatic cell count (SCC), mammary pathogens prevalence, total and specific bacterial counts, antimicrobial residues, macroscopic sediment, water addition, aflatoxins and other contaminants constitute the basis for milk payment-schemes, monitoring and improvement of flock hygiene and health management, and development of analytical surveillance programs in the dairy small ruminants. The present work reviews factors influencing the variation of these variables, including milk analytical methods, storage and preservation, along with management implications during the last two decades. Following farmer and cooperative educational programs, progressive reductions have been reported for total bacterial count and antimicrobial residue occurrence in bulk tank milk. These results were consistent, however, with high values for SCC and specific bacterial populations. Thus, mastitis control programs should be intensified to increase hygiene in milk and economic returns for producers and processors. In addition, the implementation of programs to reduce specific bacterial counts (*i.e.*, psychrotrophs, coliforms, *Clostridium* spp. spores) and mammary pathogen prevalence (*i.e.*, *Staph. aureus, Mycoplasma* spp.), as well as the use of combined screening methods for an increased rate of antimicrobial detection, are currently required strategies which are positively valuated by milk processors, industry and consumers. Other contaminants may also be present, but cost-effective screening and analytical systems have not yet been implemented. This review aims to be helpful for troubleshooting milk quality and safety, developing future premium payment systems and industry quality-standards, optimizing management, on-farm risk traceability systems and consumer acceptance.

Additional keywords: milk quality; somatic cell count; milk bacterial count; mammary pathogens; milk antimicrobial residues; water addition; aflatoxins.

Abbreviations used: AF (aflatoxins); AR (antibiotic residue); AZ (azidiol); BR (bronopol); BTM (bulk tank milk); BTMSCC (bulk tank milk somatic cell count); BTMTBC (bulk tank milk total bacterial count); CA (contagious agalactia); CCß (detection capability); CNS (coagulase-negative sptahylococci); CPO (consortium for ovine promotion); DCC (DeLaval cell counter); EU (European union); FP (freezing point); GPCNC (gram-positive catalase-negative cocci); IMI (intramammary infection); MRL (maximum residue limit); SCC (somatic cell count); TBC (total bacterial count).

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Introduction

The world production of sheep and goat milk was 28.8 million tons in 2014 (http://www.fao.org/faostat/en/#data) representing 3.6% of global milk production. Regulatory legislation including specific hygiene rules for food of animal origin, raw milk included, have been implemented by international organisms in developed countries (*i.e.*, EC, 2004), some of which have published guides on milk hygiene basically centered in cow dairy farms. Increasing awareness of public health and food safety issues in recent years has led to a greater interest in milk hygiene in small ruminant livestock in order to minimize risks and to guarantee an optimal consumption quality.

Bulk tank milk (BTM) can be contaminated by bacteria (spoiling and pathogens), bacterial and fungal toxins, veterinary drugs, cleaning and disinfectant agents, macroscopic sediment, and others, many of which are important to the farmers, cheese manufacturers and consumers because they are major factors, along with somatic cells, in determining safety and hygienic quality of the final product. Some of these variables along with fat and protein contents are the target of different legal limits or payment-by-quality schemes proposed by different countries, with obvious

repercussions on the marketing of sheep and goat milk (Pirisi *et al.*, 2007).

Bacteria contamination proceeds from different sources such as flora and pathogens present in beds, milking system and facilities, storage and transport, feeding, wash water, udder, or mastitic milk. Some of these bacteria are resistant to pasteurization, or are able to grow at refrigeration temperature, or indicate fecal contamination or mastitis, or fermenting lactic acid to butyric acid, CO_2 and H_2 causing the late-blowing defect of ripened cheeses. Despite this, only total bacterial count of BTM (BTMTBC, really bulk tank aerobic mesophilic count) has been the target of various legal limits for small ruminant milk in the European Union (EU). Other bacterial groups, such as thermoduric bacteria, psychrotrophic bacteria, coliforms, gram-positive catalase-negative cocci (GPCNC), staphylococci, or lactate-fermenting *Clostridium* spp. spores would be indicators of interest for milk hygiene, udder health, safety, and marketing quality (Arias *et al.*, 2013; de Garnica *et al.*, 2013a). Mammary pathogens (*i.e.*, *Staphylococcus* spp., *Mycoplasmas* spp., etc.) are also important organism causing mastitis and profitability leaks in the flocks, and their knowledge is essential to program specific strategies for mastitis control in each herd. *Mycoplasma agalactiae* in sheep and goats and other *Mycoplasma* spp. (*i.e.*, *M. mycoides* subsp. capri, *M. capricolum* subsp. *capricolum*, and *M. putrefaciens*) specifically in goats, are the etiological agents of contagious agalactia (CA). This chronic disease is considered endemic in many countries and has a great health and economic impact because it is associated with the typical triad of mastitis, arthritis and keratoconjunctivitis, along with pneumonia and sporadic abortion. Therefore the implementation of population control programs based on diagnostic and eradication of *Mycoplasma* spp. is very important in endemic areas (Bergonier *et al.*, 1997; Gómez-Martín *et al.*, 2013). Bulk tank somatic cell count (BTMSCC) is the first and principal tool used by technicians and farmers to evaluate udder health in flocks and to monitor the mastitis control programs and the efficiency of production processes; nevertheless, the EU has yet to regulate values in ewe and goat milk used for dairy products sold in its region. Antibiotic residue (AR) occurrence is a result of treating dairy sheep and goats with antibiotics and not withholding milk, but little attention has been paid to investigate the factors influencing its variation (*i.e.*, mastitis, antibiotic therapy, milking system, or milk production) under field conditions in these species (Gonzalo *et al.*, 2010). Other milk contaminants as aflatoxins (AF) are secondary metabolites produced by *Aspergillus* spp. that contaminate plants and foods. Stored animal feed (silage, grains and cake) are at higher risk of AF (B1 and B2) contamination. The B1 AF is carcinogenic to humans (IARC, 2002) and after its consumption by lactating animal it is biotransformed by liver into M1 AF, which is excreted into the milk. Nevertheless, the information on occurrence of AF is very scarce in small ruminants.

In addition, the accurate determination of sheep and goat milk hygiene variables is important because of economic, health, and management implications. The specific physico-chemical (dry matter contents, acidity, osmolality), synthetic (apocrine secretion, natural inhibitors), and hygienic (somatic cells, bacterial content, pathogens) characteristics of small ruminant milk compared to cow milk, as well as the preservation (azidiol: AZ, bronopol: BR, or unpreservation) and storage (refrigeration or freezing) strategies, makes necessary an analytical validation for the milk of these species in order to guarantee the global accuracy of analytical conditions and methodological procedures validated in cow milk.

As a whole, this review aims to present available knowledge about the variability of main variables defining milk hygiene in these species, to understand the relationship between these variables and to establish guidelines for monitoring purposes beginning on the premises that milk quality starts with management quality (good farming practices) in the flocks. Overall, this information would be useful firstly to milk laboratories with the objective to adapt analytical methods and strategies to sheep and goat milk, and secondly to farmers, farm organizations, milk regulatory organisms, and milk industry interested in developing milk quality standards for these species, as well as the most effective on-farm management and quality assurance programs with the objective of insuring higher quality standards and optimal milk quality and final products.

Somatic cell count

Udder health is a prerequisite to milk hygiene. Mastitis, particularly subclinical and chronic, is the most prevalent group of diseases of importance to milk hygiene in dairy livestock (Heeschen, 1987). Previous studies have confirmed that specific bacteriological examination of milk and milk SCC are reliable methods for detecting subclinical mastitis, and an inverse relationship between SCC and milk yield has been proved in meat (Fthenakis & Jones, 1990a) and dairy sheep (Gonzalo *et al.*, 1994, 2002; Leitner *et al.*, 2004a) and goats (Baudry *et al.*, 1997; Leitner *et al.*, 2004b, Pleguezuelos *et al.*, 2015).

The Fossomatic (Foss Electric, Hillerød, Denmark) and Somacount (Bentley Instruments, Chaska, MN, USA) are the most widely SCC methods used in milk-testing and interprofessional laboratories, and the performance of Fossomatic has been standardized for sheep (Gonzalo *et al.*, 1993, 2003, 2004; Martinez *et al.*, 2003) and goat (Sanchez *et al.*, 2005; Raynal-Ljutovac *et al.*, 2007) milk. Basically, Fossomatic is a DNA-specific counter based on the principle of optical fluorescence. The dye penetrates the cell and forms a fluorescent complex with the nuclear DNA. The sample is exposed to blue light, which excites the dyed cells, making these emit red light. These red light pulses are magnified, counted by a photo multiplier detector, and multiplied by the defined working factor to compute the number of somatic cells per milliliter. In cytometry on disk, the detector counts the number of cells passing by the microscope on a rotating disk. In flow cytometry, the sample is pumped through a flow cell of very small diameter that allows only one somatic cell to pass at a time. Thus, SCC by flow cytometry is slightly higher than that obtained by disk cytometry and direct microscopy (Gonzalo *et al.*, 2004) because cells aggregated in cell clusters are forced to pass in a single row through a very small capillary tube before being counted. Automation of this process means that large numbers of samples can be analyzed per hour in milk laboratories.

In sheep milk, the comparison of the Fossomatic method *vs* microscopic reference method for the different analytical conditions (Table 1) evidenced that preserved milk analyzed by flow cytometry gave optimal repeatability (s_r%=1.9 to 2.6%) and their logSCC means (5.62 to 5.67) were similar to reference value (5.63). The BR was the optimal preservative for the Fossomatic method in sheep and goat milk both in refrigeration and freezing storage (Gonzalo *et al.*, 2004; Sánchez *et al.*, 2005). The smallest SCC always corresponded to AZ-preserved milk (Gonzalo *et al.*, 2003, 2004; and Sánchez *et al.*, 2005); this SCC decrease was particularly dramatic in the defrosted milk. Analytical temperature (40ºC or 60ºC) did not contribute significantly to SCC variation in sheep (Gonzalo *et al.*, 2004) or goat (Sierra *et al.*, 2006) milks.

Fossomatic method requires, however, cumbersome and expensive equipment, so less expensive alternative methods and portable devices for on-farm or milk plant uses would be of great interest in mastitis control or quality strategies. In this sense, DeLaval cell counter (DCC, DeLaval Int. AB, Tumba, Sweden) is a portable, battery-operated optical cell counter that gives the measuring results in less than 1 min (about 40 s/sample). A cassette, which contains small amounts of fluorescent stain and Triton X-100 is used to collect milk before cell counting. A piston carries the sample toward a counting window that is exposed to a light source. Cell nuclei give fluorescent signals recorded in an image that is used to determine the SCC in milk. Similarly to cow milk, DCC showed suitable overall accuracy in goat milk compared to reference method (Berry & Broughan, 2007); nevertheless in ovine raw milk, which has a higher dry matter than goat and cow

Table 1. Mean values of log somatic cell count, standard deviation of repeatability (s_r%), coefficients of regression (b), intercept (a) and R^2 values based on linear regression analyses between direct microscopic (DM) or Fossomatic (F) reference methods and Fossomatic, DeLaval cell counter (DCC) and Bentley (B) somatic cell count (SCC) methods.

Milk	SCC methods	LogSCC	s_r%	b	a	R^2
			Regression with DM			
Sheep[1]	DM	5.63	5.22	-	-	-
	F360 (disk cytometry)	5.57-5.62	2.31-4.56	0.95-1.08	0.02 to -0.42	0.987-0.994
	F5000 (flow cytometry)	5.62-5.67	1.89-2.55	0.99-1.12	0.01 to -0.57	0.977-0.994
			Regression with F5000			
Sheep[2]	F5000	5.56	2.33	-	-	-
	DCC (0 min)	5.44	17.87	0.922	0.32	0.959
	DCC (≥ 1 min)	5.51-5.52	7.04-10.26	0.962-0.981	0.06 to 0.17	0.988-0.991
			Regression with DM			
Goat[3]	DM	5.66	-	-	-	-
	DCC (0 min)	5.56	-	1.04	-0.31	0.95
Goat[4]	B150 (flow cytometry)	-		0.97-0.98	-3 to 20	-

[1]Gonzalo *et al*. (2004). [2]Gonzalo *et al*. (2008). [3]Berry & Broughan (2007). [4]Raynal-Ljutovac *et al.* (2007).

milk, a soak time ≥ 1 min is recommended because a latent time is needed, after milk loading in the DCC cassette, for cellular membrane lysis and nuclei straining in this species (Table 1). Standard deviation of repeatability was, however, higher in DCC method than in Fossomatic method (Gonzalo *et al.*, 2006b, 2008).

At half-udder level, ewe and goat can elicit very higher SCC associated to intramammary infections (IMI) than cattle, due probably to a higher rusticity of small ruminants. Thus, SCC values > $10,000 \times 10^3$ cells/mL in case of subclinical mastitis are not infrequent in ewe and goat milk in case, for example, of mammary infections by *E. coli*, *Str. agalactiae*, *Staph. aureus*, or *Aspergillus* spp. pathogens. Cytokines played a major role in leucocyte recruitment to inflamed mammary tissue (Persson *et al.*, 1996; Persson-Waller *et al.*, 1997) and bacterial pathogens isolated in mammary infections explained a high percentage of SCC total variance in sheep (Gonzalo *et al.*, 2002). In addition, other infectious agents responsible for interstitial mastitis (*i.e.*, arthritis encephalitis virus) were also associated with a significant increase of SCC (Nord & Adnoy, 1997; Sánchez *et al.*, 2001) in goat udder halves. Regarding non-infectious factors influencing SCC variation, an important difference can be established between both species. Thus, in sheep, non-infectious factors (primary purpose, breed, flock, year, parity, lactation stage, etc.) contributed also significantly to SCC variation, but had smaller effects on SCC than bacterial infection factors (Gonzalo *et al.*, 2002; Paape *et al.*, 2007; Kern *et al.*, 2013). On the contrary, in goats, the non-infectious factors had higher importance on SCC variation, so the SCC discrimination value was lesser in this species (Luengo *et al.*, 2004; Raynal-Ljutovac *et al.*, 2007; Koop *et al.*, 2010b).

Some studies (Ariznabarreta *et al.*, 2002; Gonzalo *et al.*, 2002) allowed different infective situations to be differentiated according to SCC and isolate percentage. Isolates of main mammary pathogens and their associated SCC are shown in Table 2 for subclinical mastitis in small ruminants. Overall, goats presented higher prevalence of coagulase-positive staphylococci (22.4 *vs* 12.4%), gram-negative pathogens (6.3 *vs* 1.5%), and *Mycoplasma* spp. (3.1 *vs* 1.7%) than sheep. These three groups of organisms also elicited higher SCC than other mammary pathogens in both dairy species. Coagulase-negative staphylococci (CNS) were the most prevalent bacterial group, which elicited a large variability in SCC (4.94 to 6.27 log cells/mL in sheep, and 5.48 to 6.48 log cells/mL in goat) (Table 2). These results were consistent with the evidence that some CNS species, such as *S. simulans*,

Table 2. Isolates (mean and range, %), and logarithm of somatic cell count (mean and range, log cells/mL) associated to subclinical mastitis in dairy sheep and goat.

Organisms	Sheep				Goat			
	Isolate mean (%)	Range (%)	LogSCC mean	Range log SCC	Isolate mean (%)	Range (%)	LogSCC mean	Range log SCC
Staphylococaceae	76.6	68.6-84.9	-	-	78.4	51.4-97.7	-	-
Coagulase-negative	64.1	59.6-68.5	5.90	4.94-6.27	58.6	8.8-95.1	5.93	5.48-6.48
Coagulase-positive	12.4	4.3-25.3	6.45	6.28-6.86	22.4	2.6-72.6	6.38	5.91-6.59
Streptococcus spp.[1]	10.2	3.9-14.6	6.00	5.68-6.54	5.6	0.0-33.3	6.25	5.93-6.45
Corynebacterium spp.	4.6	0.0-13.8	5.26	5.26-6.63	2.1	0.0-24.0	5.72	5.72
Gram negative bacteria	1.5	0.6-3.9	-	-	6.3	0.0-32.0	-	-
Enterobacteria	1.4	0.0-3.9	5.40	5.40	5.3	0.0-32.0	6.63	6.50-6.88
No-Enterobacteria	0.1	0.0-0.4	6.29	6.29	1.7	0.0-7.4	6.52	6.48-6.55
Mycoplasma spp.	1.7	0.0-3.3	6.81	6.81	3.1	0.0-9.0	6.52	5.83-7.20
Other[2]	5.7	0.0-20.0	5.99	5.14-6.84	5.5	0.0-47.1	6.30	6.23-6.52
Uninfected halves	-	-	4.90	4.86-4.95	-	-	5.66	5.43-5.69
References (adapted from:)	Schoder *et al*. (1993); Fthenakis (1994); Gonzalo *et al*. (2002); Gelasakis *et al*. (2015)		Dehinhofer (1993); Schoder *et al*. (1993); Pengov (2001); Ariznabarreta *et al*. (2002); Gonzalo *et al*. (2002); Linage *et al*. (2017)		Manser (1986); Kalogridou-Vassiliadou (1991); Schoder *et al*. (1993); Poutrel *et al*. (1996); Poutrel *et al*. (1997); Contreras *et al*. (2003)		Dehinhofer (1993); Dehinhofer & Pernthaner, (1995); Poutrel *et al*. (1996); Poutrel *et al*. (1997); Sánchez *et al*. (1999); Schoder *et al*. (1993); Castro-Alonso *et al*. (2009)	

[1]Include *Enterococcus* spp. [2]Include *Bacillus* spp., *Trueperella pyogenes*, *Aspergillus* spp., unidentified organisms, etc.

S. epidermidis, *S. haemolyticus* and *S. chromogenes*, in goat and sheep, or *S. caprae* in sheep, induced high SCC (Deinhofer, 1993; Sánchez *et al.*, 1999; Pengov, 2001; Ariznabarreta *et al.*, 2002) and even caused clinical mastitis (Fthenakis & Jones, 1990b) in a way similar to the major pathogens. CNS along with other pathogens were also responsible of chronic infections associated to mammary nodular and indurative fibrous lesions in which pathogens remain for long periods. For example, in a recent study carried out in 878 ewes distributed in 10 flocks and with test-day SCC records $> 2 \times 10^6$ cells/mL, CNS were responsible of 74.9% of mammary infections, which were associated to a high prevalence (37.8%) of nodular mammary indurations evidenced by udder palpation after milking (Linage *et al.*, 2017). In spite of these facts, studies that focus on pathogenicity mechanisms of *Staphylococcus* spp. are centered mainly in coagulase-positive species (Moroni *et al.*, 2005; Linage *et al.*, 2012).

SCC test-day recording is a useful tool to monitoring subclinical mastitis and enable relevant information for effective mastitis control strategies in dairy flocks (chronic mastitis, culling, selective dry therapy, etc.). Spanu *et al.* (2011) evidenced that half-udder milk samples obtained from ewes with 3 or more test-day SCC records $\geq 400 \times 10^3$ cells/mL in the previous lactation were 5.6–7.5 times more likely to be microbiologically positive for mastitis pathogens as compared to milk samples obtained from ewes with test-day SCC records below that threshold. Therefore, identification of chronic mastitis based on test-day recording is possible and this practice should be included within mastitis control programs. In addition, test-day SCC can be used to estimate the magnitude of milk yield and economic losses. Several investigations put into question, however, the relevance of SCC as a valid tool for estimating the magnitude of milk yield losses in dairy goats (De los Campos *et al.*, 2006; Koop *et al.*, 2010b). According to these authors, the negative correlations between SCC and milk yield might be confounded with the stage of lactation effect because the inverse-lactation curve between both variables (Rota *et al.*, 1993). Therefore, a dilution effect seems to play an important role in the negative association between SCC and milk yield. Nevertheless, a more exhaustive study of the relationships between milk yield and contents and SCC in a large goat population and within stage of lactation, in order to avoid the dilution or concentration effect, evidenced important economic losses (9.5% to 31.6%) as SCC level increased within each month of lactation, from mathematical models corrected for systematic factors influencing test-day recording (Barrón-Bravo *et al.*, 2013; Pleguezuelos *et al.*, 2015). Finally, some studies suggest that a reduction in SCC can be achieved by selection in compatibility with improving milk production and udder type and teat traits in dairy sheep (El-Saied *et al.*, 1998; De la Fuente *et al.*, 2011) and goats (Rupp *et al.*, 2011).

In recent years, the discovery in the milk of a beneficial or probiotic flora with different antimicrobial properties against mammary pathogens (*i.e.*, mechanisms of competitive exclusion, production of H_2O_2 and bacteriocins, aggregation properties, etc.) has provided grounds to consider the mastitis as a dysbiosis of a milk microbial ecosystem in woman and cow milks (Espeche *et al.*, 2012; Fernández *et al.*, 2014). In this context, further investigations will be necessary to know the bacterial ecosystem in small ruminant milk and the importance of probiotic flora in the mastitis establishment.

At bulk tank level, BTMSCC is the principal tool used by technicians and farmers to evaluate and monitoring udder health in flocks. The main sources of variation of this variable have been identified in dairy sheep (Table 3). Special reference should be made to the flock factor, which explained a high percentage of BTMSCC total variance. Important differences in hygiene and management practices would bring about considerable differences in the prevalence of mammary infection and BTMSCC. In a total of 309 dairy sheep flocks of Castilla y León region (Spain), yield-weighted annual BTMSCC among flocks ranged from 250×10^3 cells/mL and $2{,}500 \times 10^3$ cells/mL. In this sense, recording of this variable can be used for monitoring flock udder health and as a criterion for milk payment schemes; nevertheless interpretation criteria are different from dairy cattle, due to increased SCC response to mammary infection of small ruminants. Although EU has yet to regulate values in ewe and goat milk, a practical approach of flock mammary health according to BTMSCC level can be established in both species: a) Good or acceptable flock mammary health with ewe/goat IMI prevalence < 25%: BTMSCC $< 750 \times 10^3$ cells/mL; b) Deficient flock mammary health with ewe/goat IMI prevalence > 50%: BTMSCC $> 1{,}500 \times 10^3$ cells/mL; and c) Intermediate situation with ewe/goat IMI prevalence between 25% and 50%: BTMSCC between 750×10^3 cells/mL and $1{,}500 \times 10^3$ cells/mL (Gonzalo *et al.*, 2000; Silanikove *et al.*, 2014). Other authors consider, however, in the case of goats, that non-infectious factors (*i.e.*, parity and stage of lactation) need to be considered when establishing legal SCC limits (Paape *et al.*, 2007). Month within herd was also a relevant factor of variation in BTMSCC that explained the variation in IMI prevalence throughout lactation in each flock, although a BTMSCC dilution effect was also possible in more productive months (*i.e.*, spring) which elicited lesser BTMSCC. Breed

Table 3. Factors influencing bulk tank somatic cell count (BTMSCC) and total bacterial count (BTMTBC) variation in dairy sheep flocks in Castilla y León region of Spain.

Source	Log BTMSCC[1]	Log BTMTBC[2]
Breed		
Awassi	6.09[a]	5.23[a]
Assaf	6.09[a]	5.22[a]
Churra	6.02[a]	5.10[b]
Castellana	5.84[b]	5.19[ab]
Milking		
Hand	6.07[a]	5.31[a]
Machine		
Bucket	6.04[a]	5.31[a]
Parlor (dead-ended milkline)	5.91[b]	5.10[b]
Parlor (looped milkline)	5.88[b]	5.01[c]
Clinical outbreak of CA[3]		
Yes	6.06[a]	5.14[a]
No	5.96[b]	5.13[a]
Antibiotic dry therapy		
Yes	5.91[b]	5.12[b]
No	6.10[a]	5.25[a]

[1]Adapted from: Gonzalo *et al*. (2005). [2]Adpated from: Gonzalo *et al*. (2006a). [3]CA: Contagious agalactia. [a,b,c]Means with different superscript in the same column (within each source) differ $p < 0.05$.

differences in BTMSCC were also evidenced associated with the milk yield level; greatest BTMSCC corresponded to more productive breeds (Table 3). In this sense, greater resistance to mastitis was reported by Dario & Bufano (1991) in less-productive breeds compared with more selected breeds, in which the IMI incidence would be probably greater. For milking type, machine milking elicited a lower BTMSCC than hand milking (Table 3); the inferior hygiene conditions of hand milking compared with machine milking makes it more difficult for farmers to reach milk quality standards as measured by BTMSCC. Within machine milking, BTMSCC was greater in bucket system than in parlor system; differences between dead-ended and looped milk lines were not statistically significant (Table 3). Moreover, high pulsation speeds and low milking vacuum levels were associated with small BTMSCC (Gonzalo *et al.*, 2005). Clinical outbreaks of CA increased BTMSCC both in dairy sheep (Table 3) and goats (Corrales *et al.*, 2004). This chronic disease is a serious limitation to improve milk hygiene in dairy flocks, therefore the implementation of programs for its prevention and eradication should be prerequisites for optimizing BTMSCC and mastitis control strategies in endemic areas. Dry therapy was other important source of variation of BTMSCC; when dry therapy was implemented BTMSCC was less than when this practice was not used, as dry therapy was efficient in reducing IMI prevalence, BTMSCC and BTMTBC, improving ewe milk hygiene (Linage & Gonzalo, 2008; Gonzalo *et al.*, 2010). Systematic dry therapy was also an efficient method for cure of subclinical mastitis and SCC control in goats, although only references at udder-half level are disposables in scientific literature (Poutrel *et al.*, 1997; Bastan *et al.*, 2015).

The evidence of high SCC (SCC test-day and BTMSCC records) in Mediterranean countries (Gonzalo *et al.*, 2005, 2010; Clément *et al.*, 2015), together with the important economic losses associated to subclinical mastitis in small ruminant flocks (Fthenakis & Jones, 1990a; Gonzalo *et al.*, 2002; Pleguezuelos *et al.*, 2015), highlight the need for implementing mastitis control programs within flocks and cooperatives, in order to improve the milk hygiene and to increase economic return for producers. Technicians and farmers must always keep in mind that mammary health of the flock is a necessary and previous condition to implementation of milk yield breeding schemes. In addition, quality assurance programs or certification schemes for dairy flocks are important tools for improving BTM quality. Because a positive relationship has been stated between BTM

quality and dairy farm audits (Velthuis & Asseldonk, 2011; de Garnica & Gonzalo, 2013), small ruminant cooperatives have currently implemented farm risk audits for monitoring mammary health in the flocks. Each farm audit record includes binary checklist items (valuated as 0: approved or 1: rejected) distributed over a number of categories or main critical areas. Each flock and each category within flock accumulates a number of risk points, which should progressively be reduced over time. Moreover, recording of BTM quality can be used to preselect high-risk flocks to be audited more frequently.

Total and specific bacterial count

Specifications for count of different bacterial groups (*i.e.*, BTMTBC, thermoduric, phychrotrophic, coliforms, GPCNC) follow American Public Health Association recommendations (White *et al.*, 1992; de Garnica *et al.*, 2011, 2013a); lactate-fermenting *Clostridium* spp. spore are enumerated by the most probable number technique in Bryant and Burkey broth specific media under anaerobiosis (Arias *et al.*, 2013) or by optimized membrane filtration technique (Reindl *et al.*, 2014); and *Staphylococcus* spp. and other mammary pathogens can be counted after culture in specific media according with de Koop *et al.* (2010a), de Garnica *et al.* (2011, 2013a), and Linage *et al.* (2012).

Storage and preservation strategies (unpreserved or AZ-preserved milk) significantly contributed to changes in microbiological variables in BTM (TBC, thermoduric, psychrotrophic, coliforms, *E. coli*, *Strep. agalactiae*, and *Staph. aureus*). Unpreserved milk stored at 4ºC increased the concentration of TBC, psychrotrophic and coliform bacteria over time. The initial concentration of thermoduric, and pathogens (*E. coli, Strep. agalactiae* and *Staph. aureus*) remained, however, invariable because probably of inhibition competitive effect of the main bacterial groups. Storage at 4ºC of AZ-preserved milk was a suitable method to maintain the initial concentrations for all bacterial groups and species, over 96 h. Freezing at -20ºC could be appropriate for analysis of mesophilic, thermoduric and psychrotrophic bacterial groups, but significantly decreased the viability of coliforms and pathogenic species in ovine milk (de Garnica *et al.*, 2011). Similarly to ovine milk, AZ-preserved samples stored at 10ºC or 4ºC were appropriate for goat BTMTBC by Bactoscan flow cytometry method for up to 24 h and 11 d postcollection, respectively. Nevertheless, refrigerated unpreserved samples showed high goat BTMTBC at 24 h postcollection (Sierra *et al.*, 2009c).

BTMTBC is a practical variable related to hygiene of milking and facilities (Table 3). The goal BTMTBC of different legal limits or payment-by-quality schemes has been proposed by different countries. For small ruminants, EU determines the limits for this variable in 500×10^3 cfu/mL in the case of non-pasteurized milk, and $1{,}500 \times 10^3$ cfu/mL when milk is to be pasteurized (EC, 2004). Both limits are, however, extremely high, indicatives of very deficient hygiene practices, and lead to a drastic reduction of milk shelf time and a poor quality of dairy products. In this sense, it is entirely possible to maintain annual average values of BTMTBC below 150×10^3 cfu/mL even in hand milked flocks if milking hygiene is appropriate and refrigeration tank is working properly (de Garnica & Gonzalo, 2013). Only very extensive livestock systems, in which the milking is carried out in areas where electrical power is not available, would justify those limits. Therefore, a reconsideration of hygiene policy about milk BTMTBC thresholds would be desirable in small ruminants, as high BTMTBC limits should be the exception rather than the rule.

The correlation between BTMTBC and BTMSCC was moderate (*i.e.*, r=0.24 to r=0.42) (Gonzalo *et al.*, 2005; Koop *et al.*, 2009; de Garnica *et al.*, 2013a) suggesting that IMI is probably an important factor driving this association in small ruminants. In this sense, programs to improve udder health (*i.e.*, dry therapy) may have a significant effect on both BTMSCC and BTMTBC (Table 3; Gonzalo *et al.*, 2010; de Garnica *et al.*, 2013a). Other bacterial groups, such as thermoduric, psychrotrophic, and coliform were significantly related to BTMTBC, whereas GPCNC and staphylococci counts were correlated with both BTMTBC and BTMSCC variables (Koop *et al.*, 2010a; de Garnica *et al.*, 2013a). Highest counts were for psychrotroph and coliform groups in dairy sheep flocks (Table 4), and a medium-high correlation (*i.e.*, r=0.51) was found between both variables (de Garnica *et al.*, 2013a), indicating that poor cleaning practices in the flocks tend to select for less-resistant organisms, such as gram-negative rods. Season was an important effect associated with the variation of BTM prevalence for specific bacterial groups and pathogens (*i.e.*, S*taph. aureus*, *E. coli*). Psychrotrophic and coliform bacterial groups (*E. coli* included) and *Staph. aureus* were highest in winter in connection with more dirty beds and udders due to the wetter weather (ambient contamination) and with beginning of milking season (contagious-pathogen infections) (de Garnica *et al.*, 2013a,b). *Staph. aureus* and *E. coli* organisms showed also higher occurrences in hand milked flocks, and *Staph. aureus* was also higher in flocks in which dry therapy was not implemented.

Table 4. Mean and standard deviation (SD) of logarithm of microbiological variable counts (log cfu/mL): staphylococcal count (SC), total bacterial count (TBC), standard plate count (SPC), psychrotrophic count (PC), coliform count (CC), thermoduric count (TC) and gram-positive catalase-negative cocci count (GPCNCC) of ewe and goat bulk tank milk.

Bulk tank milk variables	Sheep flocks (n= 205)[1]		Goat flocks (n=53)[2]	
	Mean (log cfu/mL)	SD	Mean (log cfu/mL)	SD
SC	-	-	3.88	0.35
TBC	5.13	0.42	4.53	0.19
SPC	-	-	4.55	0.43
PC	5.69	1.27	-	-
CC	3.81	1.61	1.61	0.92
TC	2.97	0.74	-	-
GPCNCC	2.95	1.88	-	-
References (adapted from:)	de Garnica *et al*. (2013a)		Koop *et al*. (2010a)	

[1]Averaged seasonal values. [2]Averaged spring values.

Psychrotrophic flora of milk becomes predominant in milk plants (*i.e.*, silo milk) exceeding 7.00 log cfu/mL from 72 h postcollection (de Garnica *et al.*, 2011). Because of this high concentration of psychrotrophic flora and its proven relationship with a high incidence of lipolytic and proteolytic activities of *Pseudomonas* spp. and *Acinetobacter* spp. on milk and cheese components (Hantsis-Zacharov & Halpern, 2007; Capodifoglio *et al.*, 2016), it is advisable to collect and process the milk (*e.g.*, delivery, heat treatment) in the shortest time possible after milking to prevent significant spoilage of milk and dairy products. This bacterial group increased with increasing total bacterial load in BTM (de Garnica *et al.*, 2013a), so it can be considered as ubiquitous in all small ruminant herds. Thermodurics are a contaminant group of milk that contains thermophilic spore-forming bacteria which can survive pasteurization during dairy-product processing causing dairy-product spoilage in the post-processing. The concentration of this group in milk is, however, generally low (Table 4). The diversity of GPCNC has been recently investigated by 16S rDNA sequencing in a total of 192 isolates from ewe BTM. This molecular approach enabled the identification of 23 species of GPCNC of sanitary and technological importance within the *Enterococcus, Streptococcus, Lactococcus, Aerococcus* and *Trichococcus* genera. Some of these species are potentially pathogenic nosocomial organisms (*i.e.*, *Enterococcus faecalis*) which have developed mechanisms of antimicrobial resistance (*i.e.*, vancomycin resistance) (de Garnica *et al.*, 2013d, 2014), so control strategies are needed in the food chain to optimize food safety in dairy products. Regarding *Clostridium* spp. spore counts in BTM, a value of 10^3 spores/L can be considered an acceptable threshold for the incentive payment systems (Pirisi *et al.*, 2007). The main risk factors are related to housing conditions, feeding characteristics and milking parlour hygiene. Indeed, several studies in ewe and goat BTM showed that the main on-farm management risk factors associated to an increase of spore counts > 10^3 spores/L were farm-made total mixed ration, the silages and wet brewer's grains used for feeding, and the presence of dust in the milking parlour (Arias *et al.*, 2013, 2016; Reindl *et al.*, 2014). Mean values found in scientific literature for ewe BTM ranged from 2.82-4.16 log spores/L (Scintu *et al.*, 2004; Garde *et al.*, 2011; Arias *et al.*, 2013), whereas maximum spore counts of 2.48 log spore/L and 3.70 log spore/L were described in goat BTM from suppliers without and with silage feeding, respectively (Reindl *et al.*, 2014).

On the whole, specific (thermoduric, psychrotrophic, coliforms, GPCNC, and *Clostridium* spp. spore) and total (SCC and TBC) BTM counts were negatively related with good-practice audit rating of the flocks (de Garnica & Gonzalo, 2013); therefore, the development of infrastructures for the improvement of BTM hygiene and flock management based on both management assurance and analytical surveillance programs, is feasible. Along with BTMTBC, some countries (*i.e.*, France) have included BTM counts of coliforms and *Clostridium* spp. spore as criteria for milk quality payment system through a combined index including these three variables (Pirisi *et al.*, 2007). In addition, the information relative to prevalence results for specific pathogens (*i.e.*, *Staph. aureus*, *E. coli*, or *Enterococcus faecalis*) or spoilage organisms (*i.e.*, *Pseudomonas* spp., *Clostridium* spp.) isolated from BTM, could be incorporated to traceability systems with the objective

of implementing preventive programs (*i.e.*, mastitis control, flock hygiene, feeding, or milk pasteurization).

Mycoplasma spp.

Control programs for CA involve monitoring milk samples to detect *Mycoplasma* spp. in endemic areas. Bacteriological analyses for *Mycoplasma* spp. identification are based on culture in solid and liquid specific media (*i.e.*, Hayflick, PPLO, pH, Eaton) incubated at 37ºC in a 5%-CO_2 humid atmosphere, along with biochemical and serological identification test, and final specific PCR-based detection assays (Poveda & Nicholas, 1998; De la Fe *et al.*, 2009; de Garnica *et al.*, 2013c). When goat milk samples with *M. agalactiae* and *M. mycoides* subsp. *capri* were subjected to different storage temperatures (refrigeration at 4°C or freezing at -20°C), preservation strategies (no preservative, AZ, or BR), and storage times at each temperature (0, 2, 4, 6, 8, 10 and 24 h at 4°C and 48 h, 1 wk, 2 wk and 4 wk postcollection at -20°C), results (Amores *et al.*, 2010) revealed that milk samples preserved with AZ could be used to detect infections caused by both species through culture-based methods. However, the sensitivity of the two *Mycoplasma* spp. to BR was different because the isolation of *M. agalactiae* in refrigerated milk samples was compromised by the presence of BR. In addition, freezing milk samples considerably reduced the viability of both *Mycoplasma* spp., although the addition of glycerol to milk samples to achieve 10% and 30% v/v solutions improved the recovery of *Mycoplasma* spp. from frozen milk samples (Boonyayatra *et al.*, 2010). In any case, to maximize the detection of these pathogens, fresh milk samples should be cultured as soon as possible, minimizing milk storage.

To improve the limitations of the bacteriological diagnosis of CA in milk samples, different PCR protocols has been described in the last years aimed to achieve diagnosis promptitude and sensitivity. So, PCR methods have been developed for *M. agalactiae* (Tola *et al.*, 1997; Marenda *et al.*, 2005; De la Fe *et al.*, 2012), for *Mycoides* spp. cluster (Manso-Silvan *et al.*, 2007; Woubit *et al.*, 2007) and for *M. putrefaciens* (Peyraud *et al.*, 2003), although PCR inhibitors in milk could diminish the usefulness of this procedure (Becker *et al.*, 2012). Validity parameters and detection limits of PCR diagnosis were related with the type of sample used (Tola *et al.*, 1996); in this sense, detection limit for *M. agalactiae* using bacteriological cultures in goat milk (10 cfu/mL with a sensitivity of 33.3%) was lower than that achieved by direct PCR in milk (10^2 or 10^4 cfu/mL, using extraction method from Tola *et al.* (1997) or commercial methods with enzymatic digestion, respectively). Because of this, the culture in the first 24 hours after samples collection is recommended in BTM with low mycoplasma concentration. Notwithstanding, in clinical mastitis samples the culture and PCR methods showed a good concordance due to higher *M. agalactiae* concentration (Tatay-Dualde *et al.*, 2015). Contrarily to mycoplasma culture, the detection of *M. agalactiae* in goat milk samples by PCR resulted unaffected both by AZ or BR addition (Amores *et al.*, 2011) and freezing of milk samples (Tatay-Dualde *et al.*, 2015). All in all, to improve the PCR sensitivity it is recommended to extract the DNA from a specific mycoplasma culture of the milk samples, instead of from the milk sample itself (Oravcová *et al.*, 2009).

Presence of *Mycoplasma* spp. in BTM and increased BTMSCC are indicative of outbreaks in flocks with clinical signs. Nevertheless, in absence of clinical symptomatology, intermittent excretion of *Mycoplasma* spp. can be evidenced in BTM when programs based on periodical samplings for their identification are carried out in endemic regions (*i.e.*, vaccinated flocks). These are flocks without clinical signs either chronically infected with mycoplasmas or that include some *Mycoplasma* spp. asymptomatic carrier, in which attenuated clinical outbreaks can be produced under certain conditions of stress or reduced host immunity (Bergonier *et al.*, 1997; Gómez-Martín *et al.*, 2013). In goats, most of the mycoplasma infected herds yielded, at least, one positive BTM sample, but in some herds with clinical cases of mycoplasmic mastitis, these pathogens could not be detected in BTM samples. In addition, the detection of *Mycoplasma* spp. in BTM samples could be achieved in any month of lactation but frequently did not persist in the next one, forcing to check different BTM samples throughout lactation to detect positive mycoplasma herds (Amores *et al.*, 2012).

Molecular pathogenesis of *Mycoplasma* spp, associated to a high and unexpected genetic variability mediated by mutation (*i.e.*, in housekeeping genes) and recombination (*i.e.*, in genes subjected to selective pressure) mechanisms, is increasingly better known in small ruminants (Flitman-Tene *et al.*, 2003; McAuliffe *et al.*, 2011; Tatay-Dualde *et al.*, 2016) and genetically engineered DNA vaccines against specific recombinant mechanisms could be developed in the near future.

Regarding the antimicrobial activity based on the *in vitro* assay, clindamycin, enrofloxacin, ciprofloxacin, danofloxacin, marbofloxacin, doxycycline, tylosin, tilmicosin and lincomycin would be appropriate antimicrobials for CA treatment. Antimicrobial resistance phenomena against *Mycoplasma* spp. have been, however, increasingly documented. Indeed, the ewe and goat field isolates were mostly resistant to

erythromycin, indicating that this macrolide would not be a suitable choice for therapy (Antunes *et al.*, 2008; de Garnica *et al.*, 2013c; Paterna *et al.*, 2013). In addition, in a recent published study carried out over the last 25 years in *M. agalactiae* isolates collected from sheep and goats in France, this species showed increased minimum inhibitory concentrations over time for several antimicrobials used against CA (Poumarat *et al.*, 2016).

At present, veterinary authorities of some CA endemic regions (*i.e.*, Castilla y León, Spain) have stablished voluntary surveillance and control programs against CA in order to classify the status and epidemiological situation of the sheep/goat livestock farms (BOCYL, 2017) and some farms have been already certified as free of CA.

Antimicrobial residues

The use of screening test is a reliable and rapid option to detect antimicrobials or chemical contaminants in milk, although in case of a conflict involving differences of criteria it will be necessary to corroborate the results with official methods (*i.e.*, HPLC) for positive samples (Kang *et al.*, 2016).

Microbial inhibitor tests

Microbial tests are routinely used to screening the presence of antibiotic residues in raw milk because they are able to detect a great number of antimicrobials in milk samples. Most current microbial inhibitor tests were initially developed to detect beta-lactam antibiotics in cow milk based on the inhibition of organism *Geobacillus stearotermophilus* var. *calidolactis*. Different studies on the sensitivity of microbial tests have been developed in the last two decades by different authors using sheep and goat milk (Contreras *et al.*, 1997; Molina *et al.*, 2003; Sierra *et al.*, 2009a,b; Comunian *et al.*, 2010; Beltrán *et al.*, 2013) and important efforts have been carried out in recent years by manufacturers to improve the performance and sensitivity of microbial tests (IDF, 2010). As a whole, microbial inhibitor tests using *Geobacillus stearotermophilus* var. *calidolactis* (Table 5) were efficient to detect the great majority of beta-lactams, some aminoglycoside (*i.e.*, neomycin and framycetin), some macrolide (*i.e.*, tylosin), and some sulphonamide (*i.e.*, sulfadimethoxine) in raw milk from small ruminants, but they were inefficient to detect other antimicrobials at concentrations lower than maximum residue limits (MRL) established by the EU (EC, 2010), such as tetracyclines, quinolones or the majority of macrolides, aminoglycosides and sulphonamides, which are usually used for therapeutic treatments in dairy livestock. In addition, individual sheep milk, free of antimicrobials, evidenced 4.8% to 10.0% of non-compliant results (false positive results) associated to increases of SCC, therefore somatic cells could be considered as natural inhibitors. This percentage of non-compliant results was lesser in the goat milk (0.6% to 4.3%) (Beltrán *et al.*, 2015b). On the other hand, the use of AZ as milk preservative caused a decrease in specificity of the microbiological methods due to the increase of inconclusive results (Montero *et al.*, 2005).

Receptor binding assays

Rapid screening test based on the use of specific receptors are currently available in flocks and milk plants to detect specific antimicrobial groups. Recently, improvements have been made in order to include different receptors in one single test; the result being combined tests capable of detecting simultaneously various groups of antibiotics. The Charm MRL BLTET (Charm Sciences Inc., Lawrence, MA, USA) is an example of immunoreceptor Rapid One Step Assay (ROSA®) that detects beta-lactam and tetracycline drugs below EU-MRL in raw milk. Detection capability at or below EU-MRL was optimal for the great majority of beta-lactams and tetracyclines in ewe and goat milk (Beltrán *et al.*, 2013), the specificity (100 minus percentage of non-compliant results obtained for individual milk free of antibiotics) being very high (97.9-100%). Very good performances (detection capability and specificity) were also reported for other receptor-binding assays, such as Betastar Combo test (Neogen Corporation, Lansing, MI, USA), SNAP Betalactam test (IDEXX Laboratories, Westbrook, ME, USA), SNAP Tetracycline test (IDEXX Laboratories), or Twinsensor^{BT} test (Unisensor, Liege, Belgium) (Beltrán *et al.*, 2014a,b). Specificity values were highest ($\geq$ 99%) in BTM compared with individual milks. In all cases, the use of AZ as milk preservative has no effect on the test response. As conclusion, receptor-binding assay can be used routinely for the antimicrobial rapid screening in small ruminant milk from the same AZ-preserved BTM samples used for analyses of fat and protein contents, SCC, TBC, water addition, etc.

In a study carried out over 5 yr from the same 209 dairy ewe flocks of Assaf breed, belonging to Consortium for Ovine Promotion (CPO), logistic regression showed significant effects of month, semester, year, BTMSCC, BTMTBC, milk yield and, particularly, dry therapy on AR variation. A monthly polynomial distribution of AR occurrence throughout the year was evidenced and inversely related to milk yield; the

Table 5. Detection capability of several microbial screening tests for the detection of antimicrobials in sheep and goat milk.

Antimicrobial	MRL[1] (µg/kg)	CCß[2] (µg/kg)				
		BRT MRL[3]	Delvotest (MCS, SP-NT and DA)[3]	Eclipse 100[3]	Copan Milk test[3]	Blue-Yellow[3]
ß-lactams[4]						
Penicillin G	4	2	2	–	2	3–4
Amoxicillin	4	3–4	3–4	3–5	3	–
Ampicillin	4	2–3	2–4	4–5	2	5–6
Benzylpenicillin	4	2–3	2–3	2–4	–	–
Cefacetrile	125	≤ 63	≤ 63	≤ 63	–	–
Cefalonium	20	15–20	≤ 10–20	15–20	–	–
Cefapirin	60	≤ 30	≤ 30	≤ 30	–	–
Deacetylcefapirin	*	≤ 30	≤ 30	≤ 30	–	–
Cefazolin	50	≤ 25	≤ 25	≤ 25	–	–
Cefoperazone	50	> 50	44– >50	> 50–97	44	> 50
Cefquinome	20	> 20	>20	>20	–	–
Ceftiofur	100	> 100	100– >100	100– > 100	–	96–107
Desfuroylceftiofur	*	110 – >100	75–100	100	–	–
Cephalexin	100	> 100	≤ 50–75	≤ 50–96	37	> 100
Cefadroxyl	*	170	57	57	57	–
Cefuroxime	*	334	44	97	44	–
Cloxacillin	30	23–27	≤ 15–23	23–42	27	33–42
Dicloxacillin	30	≤ 15	≤ 15–21	≤ 15–27	11	–
Nafcillin	30	≤ 15	7–15	≤ 15	–	–
Oxacillin	30	≤ 15	≤ 15	≤ 15	7	–
Aminoglycosides[4]						
Gentamicin	100	100 – >100	> 100	> 100	> 100	> 100
Neomycin	1,500	≤ 750–909	≤ 750	>1,500	482	915–1,084
Framycetin	1,500	–	–	–	–	720–781
Streptomycin	200	> 200	> 200	>200	> 200	> 200
Spectinomycin	200	> 200	> 200	> 200	> 200	–
Kanamycin	150	> 200	> 200	> 200	> 150	–
Macrolides[4]						
Erythromycin	40	40 – >40	> 40	> 40	> 40	> 40
Lincomycin	150	> 150	> 150	> 150	> 150	–
Tylosin	50	≤ 25–70	≤ 25	≤ 25–95	45	44–51
Tilmicosin	50	–	–	–	–	> 50
Spyramycin	200	482	> 200	> 200	> 200	> 200
Quinolones[4]						
Enrofloxacin	100	> 100	> 100	> 100	> 100	> 100
Norfloxacin	*	> 100	> 100	> 100	> 100	–
Ciprofloxacin	*	> 100	> 100	>100	> 100	–
Flumequine	50	> 50	> 50	> 50	> 50	> 50
Marbofloxacin	75	> 75	> 75	> 75	–	–
Danofloxacine	30	–	–	–	–	> 30

Table 5. Continued

Antimicrobial	MRL[1] (µg/kg)	CCß[2] (µg/kg)				
		BRT MRL[3]	Delvotest (MCS, SP-NT and DA)[3]	Eclipse 100[3]	Copan Milk test[3]	Blue-Yellow[3]
Sulphonamides[4]						
Sulfadiazine	100	> 100	≤ 50–75	≤50–75	–	–
Sulfadimethoxine	100	≤ 50 – >50	≤ 50–75	100–107	29	101–119
Sulfamethazine	100	> 100	> 100	≤ 50– > 100	121	> 100
Sulfathiazole	100	–	–	–	–	> 100
Sulfanilamide	100	> 100	> 100	> 100	> 100	> 100
Tetracyclines[4]	***100***	***> 100***	***> 100***	***> 100***	***> 100***	***> 100***
Others[4]						
Colistin	50	> 50	> 50	> 50	–	–
Trimethoprim	50	> 50	> 50	> 50	–	–
References		Sierra *et al*. (2009a,b) Beltrán *et al*. (2015b)	Sierra *et al*. (2009a,b) Comunian *et al*. (2010) Beltrán *et al*. (2015b)	Sierra *et al*. (2009a,b) Beltrán *et al*. (2015b)	Sierra *et al*. (2009a,b)	Linage *et al*. (2007)

[1]MRL = maximum residue limit established by EC Regulation 37/2010. [2]CCß = detection capability (lower antimicrobial concentration that produces at least 95% positive results). [3]BRT MRL: Analytik in Milch Produktionsund Vertriebs, Munich, Germany. Delvotest: DSM Food Specialties. Delft, the Netherlands. Eclipse 100: Zeulab, Zaragoza, Spain. Copan milk test: Copan Italia SpA, Brescia, Italy. Blue-Yellow: Charm Sciences Inc., Lawrence, MA, USA. [4]Antibimicrobial categories. *MRL non established.

highest AR occurrences were in autumn coinciding with drying-off period and with a higher frequency of antibiotic dry therapy practice in the flocks. Measurable improvements in AR occurrence would be associated with incentive, educational, audit and training programs implemented to modify the risk perception of small ruminant producers and to increase milk safety. For example, yearly occurrences drastically diminished from 2004 (1.36%) to 2016 (0.060%), corresponding the latter occurrence to < 6% of dairy sheep producers with at least 1 violation annual (CPO Annual Report, 2016, unpublished results). Because of dry therapy is an important factor influencing AR violations in dairy flocks, this practice should be standardized within a drying-off regular schedule that takes into account the separation between dry-treated and milked animals, a minimum duration (> 45 d) of dry period, and a rigorous respect of milk withdrawal period for each antibiotic treatment. Thus, mastitis control programs based on systematic dry therapy showed significant lower AR occurrences and reduced BTMSCC and BTMTBC than mastitis programs based on discontinuous or inconstant dry therapy (Gonzalo *et al.*, 2010).

Considering the frequency of use of veterinary drugs commonly applied in ovine and caprine livestock to treat and prevent mastitis, as well as the test sensitivity toward these antimicrobials at safety levels, the use of a single test allows detecting 62.8-82.4% of the antibiotics employed. However, the simultaneous use of two screening tests with a different analytical basis (*i.e.*, microbial inhibitor test and receptor-binding assay) increased significantly the total detection range (81.5-90.1% for sheep and 84.7-92.6% for goats), reaching values ≥ 90% in some cases. In addition, the periodical use of screening tests able to detect quinolones, macrolides or aminoglycosides would be recommended to improve the screening effectiveness and to ensure the safety of milk and dairy products from sheep and goats (Beltrán *et al.*, 2015a). This is important, for example, in the case of CA endemic areas (*i.e.*, Mediterranean countries), because the antibiotics of choice for the treatment of this syndrome are quinolones and macrolides.

Water addition

Water addition is an infringement that can be considered as a hygiene problem if water is unsafe or has an unknown origin. The addition of water affects the freezing point (FP) of milk so it is used in the industry as a criterion for economic sanction. Although sheep and goat FP is influenced by many factors (*i.e.*, total non-fat solids, breed, stage of lactation, season, and pasteurization) (Antunac *et al.*, 2001; Park *et al.*, 2007; Mayer & Fiechter, 2012; Janstová *et al.*, 2013), both

analytical methodology and preservation strategies have major effects on inter- and intra-laboratory quality testing, as well as for routine sampling strategy. Indeed, FP obtained by Milkoscan FT 6000 has a high repeatability and reliable accuracy compared to thermistor cryoscope method, which is the reference method according to IDF (2002). Thus, Sánchez *et al.* (2007) suggested that Milkoscan method can be used for screening purposes in BTM samples under different analytical conditions. Moreover, within each method, the milk FP must be interpreted by taking into account the type and concentration of preservative used, as the effect of the preservative could lead to errors regarding to added water percentage. The best analytical condition for FP determination using Milkoscan FT 6000 involved the preservation with BR 0.2 g/L, while the increase in the concentration of sodium azide in the AZ formula contributed to an important reduction in the FP recorded (Sánchez *et al.*, 2007). Limits of -540 mºC in goat milk, and -560 mºC in ewe milk have been proposed for the detection of added water, indicating a good ability of the instrumental method for FP screening in milk of both species. Besides the intentional addition of water into milk, the water of milking machine final rise, which remains in pipelines of milking installation, is the main cause of water presence in milk. Therefore, an adequate slope of milk pipelines toward the final unit, as well as a proper water drainage before milking are essential to prevent this problem.

Macroscopic sediment

Sediment in milk is associated generally with poor milking hygiene procedures and environmental conditions, and filtering methodologies have been developed to quantity the sediment level in milk. Milk sediments can get stuck in very small capillary tube of flow cytometers and cause failures or malfunctions of automatic analytical devices in milk laboratories. To avoid this fact, many laboratories do not analyze BTM with macroscopic sediments or from flocks with deficient hygiene conditions, and this creates problems between industry and farmers. An appropriate education and training in cleanliness practices (*i.e*, animals, environment, milk filters maintenance or renewal, milk tank cleaning, etc.) is needed in such cases.

Others

Other microbiological (*i.e.*, verotoxigenic *E. coli, Listeria monocytogenes, Salmonella* spp.), chemical (*i.e.*, aflatoxins, pesticides) or prions (*i.e.*, scrapie) milk contaminants are possible and must be prevented and suppressed. In this sense, milk testing and interprofessional laboratories should include appropriate analytical methods for traceability and control; nevertheless, cost-effective screening and analytical systems have not yet been implemented for a great majority of contaminants. In the case of AF, screening commercial ELISA (Enzyme-Linked ImmunoSorbent Assay) kits are available for milk M1 AF quick detection. The Íscreen Afla M1 test kit (Tecna s.r.l., Trieste, Italy), the Aflatoxin M1 test kit (Euro-Diagnostica B.V, Arnhem, The Netherlands), the Transia Plate Aflatoxin M1 (Raisio Diagnostics SAS, Lyon, France), or the Ridascreen Aflatoxin M1 30/15 test kit (R-Biopharm AG, Darmstadt, Germany) could be reliable options to obtain acceptable results for screening M1 AF in small ruminant milk, the recovery percentages being between 78% and 130% for a range of M1 AF in milk between 25 and 100 ng/kg (Rubio *et al.*, 2009a). The combined use of refrigeration and preservation (AZ or BR) could slightly interfere the recovery of M1 AF (Rubio *et al.*, 2009b), while freezing of unpreserved milk did not modify the accuracy of ELISA tests for this AF (Rubio *et al.*, 2008). Receptor-binding assays (*i.e.*, Charm MRL Afla M1; Charm Sciences Inc., Lawrence, MA, USA) are also used in ovine and caprine milk plants to detect M1 AF at EU-MRL concentration (50 ng/kg) in 15 min. Results published on occurrence of AF in large small ruminant populations are scarce; for example, in a study to test M1 AF involving 407 BTM samples from farms and 82 silo milk and curd samples from cheese factories, collected from southeast Spain during 2 yr, 99.3% of the bulk tank samples and 98.8% of the silo milk and curd samples from cheese factories had M1 AF levels below EU-MRL for milk (Rubio *et al.*, 2011).

Conclusions

In small ruminants, milk hygiene has serious economic and sanitary consequences for farmers, dairy industry and consumers due to its relationship with milk payment-schemes, production loses, discarded milk and environmental pollution, profitability and operational cost, milk lifetime, manufacturing properties and marketing, food safety and other effects on final product. Milk quality is the product of a global concept of animal production, which implies the need of using specific infrastructures for its surveillance. Analytical validation for the milk of these species is needed in order to guarantee the global accuracy for different milk variables and to interpret correctly the analytical results. Suitable control strategies based on quality assurance and

analytical surveillance programs and periodical audits within herds would be the best guarantee to prevent high SCC, pathogens and indicator bacterial groups, occurrence of antimicrobial residues, aflatoxins, and other contaminants in BTM, as well as to increase the economic returns for producers and processors. Further investigations will be necessary to know in deep the balance established between beneficial and pathogenic bacteria within the milk microbial ecosystem, as well as their possible application to development of non-antibiotic mastitis control strategies in small ruminants. In addition, new cost-effective screening and analytical systems should be developed to detect a higher number of contaminants in small ruminant milk. As a whole, a better understanding of milk hygiene variables will allow producers to adopt production practices that will result in a safer and higher-quality milk that meets goal quality standards for industry and consumer acceptance.

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