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Effect of Freezing on Fossomatic Cell Counting in Ewe Milk

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ABSTRACT

Using the Fossomatic method, a total of 10,072 analytical somatic cell count (SCC) observations were carried out on 4760 aliquots taken from 70 individual ewe milk samples with the objective of studying whether freezing showed significant differences of SCC in comparison with refrigeration, according to different analytical conditions. These conditions were four preservation procedures (without preservation, potassium dichromate, azidiol, and bronopol), two storage temperatures (refrigeration and freezing), five milk ages within storage (24 h postcollection in refrigeration, and 24 h, 15, 30, and 60 d postcollection in freezing), two thawing types (rapid and slow), and two analytical temperatures (40 and 60°C).

Preservation, storage, and analytical temperature, type of thawing and milk age within storage, and most of the interactions showed a significant effect on the SCC variation. On average, the SCC was lower after freezing than in refrigeration. This effect depended specifically on type of preservation and analytical temperature of milk. The SCC of milk unpreserved or preserved with bronopol or potassium dichromate, and analyzed at 40°C, was not affected by freezing; however, use of azidiol as a preservative before freezing, and heating the milk to 60°C following thawing resulted in significantly decreased SCC. Milk age had little quantitative influence on SCC of thawed milk. The type of thawing (rapid and slow) did not significantly influence SCC of milk analyzed at 40°C. As a result, when using properly handled samples, the Fossomatic method could be used to enumerate SCC in samples frozen over the 60 d.
(**Key words:** ewe milk, somatic cell count, Fossomatic method, frozen milk)

Abbreviation key: AZ = azidiol, **BR** = bronopol, **FT** = freezing temperature, **PD** = potassium dichromate, **RT** = refrigeration temperature, **WP** = without preservative.

INTRODUCTION

In dairy sheep (González-Rodríguez et al., 1995; Gonzalo et al., 2002) SCC is a useful predictor of IMI and, therefore, an important component of milk with regard to aspects of quality, hygiene, and mastitis control. Accuracy of milk SCC is very important to most dairy farmers and to the dairy industry. The Fossomatic is the most widely used SCC method in milk-testing laboratories and its performance has been completely standardized for cow milk (Heald et al., 1977; IDF, 1995; Schmidt-Madsen, 1975, 1979). In ewe milk, which has a higher content of total solids than cow milk, the performance of Fossomatic method has been evaluated by some authors (Gonzalo et al., 1993), and its optimal analytical conditions (type of preservation, analytical temperature, and milk age) have recently been defined for refrigerated and stored at ambient temperature milk (Gonzalo et al., 2003).

Basically, the Fossomatic is a DNA-specific counter based on optical principle of fluorescence (IDF, 1995). The ethidium bromide dye penetrates the cell and forms a fluorescent complex with the nuclear DNA. Each cell produces an electrical pulse, which is amplified and recorded. Automation of this process means that large numbers of samples can be analyzed per hour in milk-testing laboratories. In most cases, Fossomatic counters use fresh or preserved milk. Very few studies have been carried out on their performance with frozen milk (Barkema et al., 1997). Freezing milk could be useful in the event of a breakdown in SCC equipment, or in biological or bacteriological protocols for frozen milk samples. However, very little is known about SCC variation factors (perservative, type of thawing or analytical temperature) in thawed ewe milk (Gonzalo et al., 1993).

The objectives of this study were to assess the suitability of frozen milk for SCC determination by the Fossomatic method, using refrigerated milk as the gold standard, and to define the influence of preservation, thawing conditions, analytical temperature, and duration of freezing on the SCC.

MATERIALS AND METHODS

A total of 70 individual ewe milk samples (250 ml/ewe), ranging between 15×10^3 and 6500×10^3 cells/

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Table 1. Study design from 70 ewe milk samples (68 aliquots/ewe sample) using a schematic diagram.

Preservation	Storage	Sample age (post collection)	Type of thawing	Analytical temp.
			—	40 and 60°C
17 aliquots without	1 at 4°C	24 h	Rapid (2 aliquots/age)	40 and 60°C
preservative	16 at –20°C	24 h, 15, 30, and 60 d	Slow (2 aliquots/age)	40 and 60°C
			—	40 and 60°C
17 aliquots with	1 at 4°C	24 h	Rapid (2 aliquots/age)	40 and 60°C
potassium dichromate	16 at –20°C	24 h, 15, 30, and 60 d	Slow (2 aliquots/age)	40 and 60°C
			—	40 and 60°C
	1 at 4°C	24 h	Rapid (2 aliquots/age)	40 and 60°C
17 aliquots with azidiol	16 at –20°C	24 h, 15, 30, and 60 d	Slow (2 aliquots/age)	40 and 60°C
			—	40 and 60°C
	1 at 4°C	24 h	Rapid (2 aliquots/age)	40 and 60°C
17 aliquots with bronopol	16 at –20°C	24 h, 15, 30, and 60 d	Slow (2 aliquots/age)	40 and 60°C

ml, were divided into 68 3-ml aliquots, which, in turn, were divided into four groups according to the preservative used: 17 without preservative (**WP**), 17 with potassium dichromate (**PD**) (0. 1 g/100 ml), 17 with azidiol (**AZ**) (0.024 g sodium azide/100 ml) and 17 with bronopol (**BR**) (0.05 g/100 ml). As shown in the schematic diagram in Table 1, one aliquot out of each group was stored at refrigeration temperature (**RT**) (4°C) and the other 16 were stored at freezing temperature (**FT**) (–20°C). The refrigerated aliquots were analyzed at 40 and 60°C, 24 h postcollection, and were used as reference. Aliquots were heated successively in a water bath increasing water temperature from 40 to 60°C after the first analysis, according to Gonzalo et al. (2003); so only one aliquot/preservation type was used (Table 1). The 16 frozen ones were analyzed by Fossomatic method 24 h, 15, 30, and 60 d postcollection, at a rate of four aliquots/age. Two types of thawing were used for each age: slow and rapid. Slow thawing consisted of keeping two aliquots in a nonshaking water bath at 18°C for 45 min. One of them was then heated to 40°C and the other to 60°C, both for 20 min, before being analytically determined. The other two aliquots were subjected to rapid thawing by placing them in nonshaking water baths at 40 and 60°C, respectively. The SCC analyses started 20 min later. All the SCC analyses were in duplicate using a Fossomatic 90 (A/S N Foss Electric, Hillerøød, Denmark) and following the previously described method (Gonzalo et al., 1993, 2003; IDF, 1995). The total number of SCC analytical observations came to 10,072.

Statistical Analysis

The statistical study was carried out following the GLM procedure of SAS (SAS, 1992). In the model used for this study, the effect of ewe was random and the others were fixed:

$$Y_{ijklmnr} = \mu + E_i + S_j + P_k + T_l + SP_{jk} + ST_{jl} + PT_{kl} + SPT_{jkl} + A_{m(j)} + D_{n(j)} + PA_{km(j)} + PD_{kn(j)} + TA_{lm(j)} + TD_{ln(j)} + AD_{mn(j)} + PAD_{kmn(j)} + TAD_{lmn(j)} + TPA_{lkm(j)} + TPD_{lkn(j)} + e_{ijklmnr}$$

where:

Y_{ijklmr} = dependent variable logSCC;
μ = mean;
E_i = ewe effect (70 levels);
S_j = storage temperature effect (two levels: RT and FT);
P_k = preservation effect (four levels: WP, PD, AZ and BR);
T_l = analytical temperature effect (two levels: 40 and 60°C);
SP_{jk} = storage × preservation interaction;
ST_{jl} = storage × analytical temperature interaction;
PT_{kl} = preservation × analytical temperature interaction;
SPT_{jkl} = storage × preservation × analytical temperature interaction;
$A_{m(j)}$ = effect of milk age within storage. Ages considered were 24 h at RT and 24 h, 15, 30 and 60 d at FT;
$D_{n(j)}$ = effect of thawing type within storage. Two types of thawing were considered: rapid and slow;
$PA_{km(j)}$ = preservation × age interaction within storage;
$PD_{kn(j)}$ = preservation × thawing type interaction within storage;
$TA_{lm(j)}$ = analytical temperature × age interaction within storage;
$TD_{ln(j)}$ = analytical temperature × thawing type interaction within storage;
$AD_{mn(j)}$ = age × thawing type interaction within storage;
$PAD_{kmn(j)}$ = preservation × age × thawing type interaction within storage;
$TAD_{lmn(j)}$ = analytical temperature × age × thawing type interaction within storage;

Table 2. Analysis of variance of the variation factors studied for logSCC variable.

Source of variation	df	*F*
Ewe	69	1220.1***
Storage temperature	1	1012.1***
Preservation	3	697.7***
Analytical temperature	1	1014.4***
Storage × Preservation	3	151.6***
Storage × analytical temperature	1	1049.9***
Preservation × analytical temperature	3	19.7***
Storage × preservation × analytical temperature	3	23.8***
Age of milk within storage	3	4.5**
Type of thawing within storage	1	107.5***
Preservation × age within storage	9	1.4^{NS}
Preservation × thawing type within storage	3	5.9***
Analytical temperature × age within storage	3	6.8***
Analytical temperature × thawing type within storage	1	132.4***
Thawing type × age within storage	3	2.6*
Preservation × thawing type × age within storage	9	0.5^{NS}
Analytical temperature × thawing type × age within storage	3	1.5^{NS}
Preservation × analytical temperature × age within storage	9	0.5^{NS}
Preservation × analytical temperature × thawing type within storage	3	9.5***

$^{NS}P > 0.05$.
$^{*}P < 0.05$.
$^{**}P < 0.01$.
$^{***}P < 0.001$.

$TPA_{lkm(j)}$ = analytical temperature × preservation × age interaction within storage;
$TPD_{lkn(j)}$ = analytical temperature × preservation × thawing type interaction within storage; and
$e_{ijklmnr}$ = random residual.

RESULTS AND DISCUSSION

Log10SCC mean of analytical observations was 5.39 ± 0.005. Table 2 shows the analyses of variance for the factors affecting logSCC. Ewe, storage temperature, preservation, analytical temperature, age of milk within storage, type of thawing within storage, and most of the interactions of the previously mentioned effects contributed significantly to the SCC variation.

Regardless of storage factor, preservation and analytical temperature were important logSCC variation factors ($F = 698$ and 1014, respectively), as well as interaction of both (Table 2). The highest values were obtained for BR preserved milk (5.52 ± 0.005) and the lowest ones for AZ preserved milk (5.34 ± 0.005), coinciding with a recent study on RT stored ewe milk (Gonzalo et al., 2003). However, the analytical temperature of 60°C caused a significant decrease ($P < 0.001$) in log SCC (5.42 ± 0.003) in comparison with that of 40°C (5.48 ± 0.003), contrasting with results obtained by other authors in refrigerated cow (Miller et al., 1986) and ewe milk (Gonzalo et al., 2003). Biological significance of this discrepancy will later be explained after analyzing interaction of temperature with other effects.

The storage effect (RT and FT) showed high statistical significance ($F = 1012$; $P < 0.001$); logSCC was significantly higher ($P < 0.001$) at RT (5.52 ± 0.004) than FT (5.37 ± 0.001), in agreement with results of other cow (Barkema et al., 1997) and ewe (Gonzalo et al., 1993) milk studies. Interactions of storage with the previous effects were significant too ($P < 0.001$): storage × analytical temperature ($F = 1050$), storage × preservation ($F = 152$), and storage × preservation × analytical temperature ($F = 24$). Therefore, storage factor must be analyzed considering type of preservation and analytical temperature at the same time.

Age and type of thawing, both within storage, were also significant. Age within storage ($F = 4.5$; $P < 0.01$) showed little quantitative importance (Table 3); least squares means of logSCC in frozen milk were 5.37 on d 1, 15, and 60, and 5.38 on d 30 postcollection. Small variations of logSCC over time were also found by Barkema et al. (1997) in thawed cow milk in accordance with our results. However, type of thawing showed high

Table 3. Least squares means of logSCC by age of freezing.

Age (freezing)	Mean	SE	SCC[1]
24 h	5.37^{b}	0.003	233
15 d	5.37^{b}	0.003	232
30 d	5.38^{a}	0.003	240
60 d	5.37^{b}	0.003	234

^{a,b}Means in the same column with different superscripts differ $P < 0.05$.
[1]Geometric mean ($\times 10^3$/ml).

Table 4. Least squares means of logSCC by analytical temperature × thawing type interaction within storage.

Analytical temperature	Refrigeration		Rapid thawing		Slow thawing	
	Mean[1]	SCC[3]	Mean[2]	SCC[3]	Mean[2]	SCC[3]
40°C	5.48	300	5.48	300	5.48	303
60°C	5.57[a]	370	5.30[b]	198	5.23[c]	169

[a,b,c]Means in the same row with different superscripts differ $P < 0.05$.

[1]SEM = 0.006.

[2]SEM = 0.003.

[3]Geometric mean (× 10^3/ml).

statistical significance ($F = 108$; $P < 0.001$); rapid thawing showed higher values (5.39 ± 0.002) than slow thawing (5.35 ± 0.002). Milk is a colloidal suspension and so these differences of SCC could be due to the different effect of thawing conditions on aqueous and fat phases of milk. Because SCC is more associated with the fat fraction, SCC results may get skewed due to nonhomogeneous or mixed samples. In addition, thawing also showed significant interactions with preservation ($F = 5.9$; $P < 0.001$) and especially with analytical temperature ($F = 132$; $P > 0.001$) within storage. Thus, whereas at 40°C there were no significant differences ($P > 0.05$) of logSCC means between refrigeration (5.48), rapid (5.48), and slow (5.48) thawing, such means significantly differed ($P < 0.001$) at 60°C (5.57, 5.30, and 5.23, respectively) (Table 4).

Finally, type of thawing × preservation × analytical temperature interaction within storage was highly significant ($F = 9.5$; $P < 0.001$) and enabled the previously mentioned effects to be studied more suitably, considering for this aim the least squares means that are shown on Table 5. To compare these means, it is necessary to bear in mind that SCC of milk BR preserved RT stored and analyzed at 40°C must be considered as a reference value. In fact, Gonzalo et al. (2003) found that these conditions elicit the most accurate SCC compared with the reference microscopic method (IDF, 1995). Thus, at 40°C, logSCC of BR preserved milk was independent ($P > 0.05$) of storage temperature and type of thawing (5.53, 5.55, and 5.54 for refrigeration and rapid and slow thawing, respectively). At such temperature (40°C) thawed WP or PD-preserved milk also showed logSCC values (5.51 to 5.54) similar ($P > 0.05$) to those of RT-stored BR preserved milk (Table 5). However, SCC noticeably decreased ($P < 0.001$) after thawing in AZ preserved milk (5.31 and 5.33 after rapid and slow thawing, respectively) (Table 5). The log SCC of refrigerated milk analyzed at 60°C (5.55 to 5.99) were higher ($P < 0.001$) than those found at 40°C (5.44 to 5.53), coinciding with results obtained by Gonzalo et al. (2003) for ewe milk, probably because higher temperatures favor better penetration of the ethidium bromide dye in the cell, or because it disperses the fat in ewe milk better. However, log SCC decreased very significantly

Table 5. Least squares means of logSCC by preservation × analytical temperature × thawing type interaction within storage.

Storage	Preservation	40°C		60°C	
		Mean	SCC[1]	Mean	SCC[1]
Refrigeration[2]	BR	5.53[ab]	338	5.59[a]	386
	PD	5.48[c]	303	5.57[ab]	369
	WP	5.44[d]	275	5.57[ab]	373
	AZ	5.46[cd]	286	5.55[b]	353
Rapid thawing[3]	BR	5.55[a]	353	5.45[c]	280
	PD	5.54[a]	347	5.35[d]	225
	WP	5.52[b]	327	5.30[e]	200
	AZ	5.31[e]	203	5.09[f]	122
Slow thawing[3]	BR	5.54[a]	350	5.39[g]	243
	PD	5.54[a]	348	5.33[h]	212
	WP	5.51[b]	326	5.21[i]	162
	AZ	5.33[f]	211	4.99[j]	97

[a,b,c,d,e,f,g,h,i,j]Means in the same column with different superscripts differ $P < 0.05$.

[1]Geometric mean (× 10^3/ml).

[2]SEM = 0.013.

[3]SEM = 0.006.

($P < 0.001$) in milk stored at FT and analyzed at 60°C (4.99 to 5.45) (Table 5). The sum of the effects of thawing and heating to 60°C could have too traumatic an effect on the cells and affect their capacity to fix the ethidium bromide dye, or lead to samples in which aqueous and fat phases were nonhomogeneous. At this temperature, rapid thawing showed significantly higher ($P < 0.05$) logSCC (5.09 to 5.45) than slow thawing (4.99 to 5.39), whereas at 40°C no statistical differences were recorded between both types of thawing (5.31 to 5.55 for rapid thawing and 5.33 a 5.54 for slow thawing). The lowest values of logSCC corresponded to AZ preserved milk analyzed at 60°C after thawing (5.09 and 4.99) (Table 5).

Therefore, the previous results allow us to state that samples frozen up to 60 d postcollection but handled properly and analyzed at 40°C gave similar SCC to the BR refrigerated reference.

CONCLUSIONS

The FT storage of BR or PD preserved milk offered ideal conditions for cellular integrity. On the other hand, AZ preservation was definitely not advised for freezing milk to be used in SCC analysis. The optimum analytical temperature after thawing was 40°C, as SCC decreased significantly at 60°C. Sample age was of less quantitative importance on SCC; logSCC means remained relatively constant over the 60 d of this study. Rapid or slow thawing did not significantly influence SCC of milk analyzed at 40°C. As a result, from a practical point of view, WP milk, and BR- or PD-preserved milk before freezing and analyzed at 40°C after rapid or slow thawing elicited SCC values similar to those of refrigerated milk at 24 h.

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