Short communication: Effect of storage and preservation on total bacterial counts determined by automated flow cytometry in bulk tank goat milk

D. Sierra,* A. Sánchez,† A. Contreras,†¹ C. Luengo,* J. C. Corrales,† C. de la Fe,† I. Guirao,* C. T. Morales,* and C. Gonzalo‡

*Laboratorio Agroalimentario y de Sanidad Animal, Consejería de Agricultura y Agua, Comunidad Autónoma de la Región de Murcia, El Palmar, 30120 Murcia, Spain

†Departamento de Sanidad Animal, Facultad de Veterinaria, Universidad de Murcia, 30071 Murcia, Spain

‡Departamento de Producción Animal, Facultad de Veterinaria, Universidad de León, 24071 León, Spain

ABSTRACT

This study was designed to evaluate the effects of different storage conditions on total bacterial count (TBC) determinations made in goat bulk tank milk using an automated flow cytometry method. The storage conditions tested were storage temperature (refrigeration at 4 and 10°C or freezing at –20°C), the use of a preservative (no preservative, NP; azidiol, AZ; or bronopol, BR), and the age of the milk samples for each analytical condition (storage times at 4°C: from 0 h to 5 d for NP; and from 0 h to 22 d for AZ and BR; storage times at 10°C: from 24 h to 2 d for NP and from 24 h to 22 for AZ and BR; storage times at –20°C: from 24 h to 22 d for NP, AZ, and BR). Significant effects on individual bacterial count (IBC) variation were shown by the bulk tank milk sample, preservative, storage temperature, interaction preservative × storage temperature, and milk age within the interaction preservative × storage temperature. In preserved samples, the highest IBC were obtained for AZ and the lowest counts were obtained in samples preserved with BR. Because of the variation in IBC recorded in BR-preserved samples, we recommend that BR should not be used for TBC determinations using the automated flow cytometry method. The NP samples stored at 4 and 10°C showed significantly higher IBC at 24 h postcollection, also invalidating these analytical conditions for TBC analyses. The practical implications of our findings are that goat milk samples preserved with AZ and stored at 10 or 4°C are appropriate for TBC by the BactoScan flow cytometry method for up to 24 h and 11 d postcollection, respectively.

Key words: goat milk, total bacterial count, BactoScan flow cytometry, milk preservative

The total bacterial count (TBC) of bulk tank milk is an important tool used to monitor milk quality in dairy goats and sheep (Muehlherr et al., 2003). Because of its implications for milk quality and food safety, the TBC is regulated according to the official milk standards of the different countries. In the European Union, the limits established (Regulation EC No. 853/2004; European Union, 2004) for sheep and goat milk used in dairy products are $1,500 \times 10^3$ cfu/mL for milk subjected to heat treatment and 500×10^3 cfu/mL for milk not subjected to heat treatment. In countries outside Europe, including the United States, TBC limits established for goat and sheep milk are the same as for cow milk (i.e., 100×10^3 cfu/mL in the United States; PMO, 2007).

In milk-testing laboratories, the TBC is routinely determined using automated flow cytometry (FC) devices. In the FC method, the bacteria are stained with a specific DNA/RNA fluorescent dye (ethidium bromide) and counted by the capture of the light pulses excited by a laser source into a flow cell. The FC technique expresses the TBC results in units defined as individual bacterial counts (IBC), which need to be converted into the scale of the reference method (cfu/mL). Several authors have explored the relationship between IBC and colony-forming units (Ninane et al., 2000; Suhren and Walte, 2000; Bolzoni et al., 2001; Tomaska et al., 2006).

Currently, preservatives are added to milk samples before testing for payment-by-quality schemes, official control of milk quality, and dairy herd improvement programs. Several preservation strategies are used for milk samples destined to undergo SCC using fluoro-optoelectronic counters (IDF, 2006) or the determination of milk fat, protein, and lactose contents by mid-infrared measurements (IDF, 2000). The effects of these preservatives on goat milk SCC, composition (Sánchez et al., 2005), and milk freezing-point determinations (Sánchez et al., 2007) have also been assessed. Analytical conditions affecting automatic TBC determinations have been evaluated in cow (Barcina et al., 1987; Suhren and

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¹Corresponding author: acontrer@um.es

Table 1. Composition and total bacterial counts (individual bacterial count, IBC, and cfu/mL) of the bulk tank milk samples (analyses were performed in duplicate)

Item	n	Mean	SD	Minimum	Maximum
Freezing point, m°C	35	-557	2.8	-552	-561
SCC, $\times 10^3$ cell/mL	35	1,638	180	1,374	1,971
Nonfat dry matter, %	35	8.883	0.102	8.660	9.010
DM, %	35	14.658	0.614	13.560	15.490
Fat, %	35	5.693	0.517	4.840	6.380
Total protein, %	35	3.530	0.054	3.420	3.590
Lactose, %	35	4.618	0.045	4.510	4.680
pH	35	6.695	0.019	6.670	6.740
LogIBC/mL	35	6.582	0.497	5.710	7.297
Log cfu/mL ¹	35	5.930	0.386	5.255	6.486
Colony-forming units, $\times 10^3$ /mL	35	1,181	832	180	2,982

¹Colony-forming units were calculated using the model defined by Spanish government: $y = 0.776x + 0.823$, where $y = \log \text{cfu/mL}$ and $x = \log \text{IBC/mL}$ (Ministerio de la Presidencia, 2008).

Walte, 2000) and sheep milk (Elizondo et al., 2007). However, the most adequate conditions for the instrumental determination of TBC in goat milk have not yet been determined and neither have the main sources of analytical variation in this species.

The present study was designed to determine the effects of storage temperature, storage time, and preservative used on TBC values, and its repeatability, obtained by automated FC in bulk tank samples of goat milk.

Thirty-five 1,500-mL bulk tank milk samples were obtained from 35 herds of machine-milked Murciano-Granadina goats. All samples were kept at 4°C and immediately transported to the laboratory (Laboratorio Agroalimentario y de Sanidad Animal, Murcia, Spain) where aliquots were immediately prepared. Each sample was divided into eighty 18-mL aliquots ($n = 2,800$), using a 34×43 mm polyethylene container, and 79 were assigned to different experimental groups. The 3 preservative treatments tested were no preservative (NP), azidiol (AZ), or bronopol (BR). For each preservative treatment, 10 aliquots each were stored at 10 and -20°C, and 11 aliquots were stored at 4°C. However, for the NP treatment, we tested only 2 aliquots stored at 10°C, 5 aliquots at 4°C, and 10 aliquots at -20°C. The storage times tested at 4°C were 0 and 24 h, and 2, 3, and 5 d for NP and 0 and 24 h, and 2, 3, 5, 7, 9, 11, 14, 18, and 22 d for AZ and BR. The storage times considered at 10°C were 24 h and 2 d for NP, and 24 h and 2, 3, 5, 7, 9, 11, 14, 18, and 22 d for the other 2 preservation treatments. The storage times tested at -20°C were 24 h and 2, 3, 5, 7, 9, 11, 14, 18, and 22 d for NP, AZ, and BR. For the preparation of AZ, 1.8 g of sodium azide, 0.075 g of chloramphenicol, 1 mL of ethanol, and 4.5 g of sodium citrate pentahydrate were dissolved in 100 mL of distilled water, to which 0.035 g of bromophenol blue was added as a colorant. Bronopol was prepared by dissolving 5 g of 2-bromo-2-nitro-1,3-

propanediol (Sigma-Aldrich, St. Louis, MO; reference 134708) in 100 mL of distilled water and adding 0.05 g of methylene blue as a colorant. Preservatives were added at 80 $\mu\text{L}/20$ mL of milk for BR (final milk concentration of 0.020 g/100 mL) and at 66 $\mu\text{L}/20$ mL of milk for AZ (final milk concentration of 0.006 g of sodium azide/100 mL).

In one aliquot of each bulk tank milk sample, milk composition and SCC were determined in duplicate using a CombiFoss 6000 FC apparatus (Foss Electric, Hillerød, Denmark). Table 1 shows the composition of the 35 original bulk tank milk samples used. A microbiological inhibitor test (Delvotest MCS, DSM Food Specialties, Delft, the Netherlands) was used to screen for antimicrobial drugs in these samples.

In all aliquots, TBC were determined in duplicate (2 consecutive determinations on each aliquot) using an automated flow cytometer (BactoScan FC, Foss Electric). Following Cassoli et al. (2007), results were expressed in individual bacterial counts per milliliter and log-transformed. Before and during the experiments, the equipment was subjected to interlaboratory quality control tests by a reference laboratory (J. Hüfner, Hergatz, Germany).

Comparison of the least squares means was carried out using the GLM procedure implemented in the SAS 6.11 software package (SAS Institute, 1996) according to the model, in which all the effects studied were fixed:

$$Y_{ijkl} = \mu + B_i + S_j + P_k + SP_{jk} + A_{l(jk)} + e_{ijkl},$$

where Y_{ijkl} = the dependent variable, that is, logIBC/mL; μ = the mean; B_i = the effect of the bulk tank milk sample ($n = 35$); S_j = the effect of the storage temperature (3 levels: 4, 10, and -20°C); P_k = the effect of the preservative (3 levels: NP, AZ, and BR); SP_{jk} = the effect of the interaction storage temperature $\times$

Table 2. Analysis of variance of variations in logIBC/mL¹

Source of variation	df	F	P-value
Bulk tank milk sample	34	1,331.70	<0.0001
Preservative	2	10,755.80	<0.0001
Storage temperature	2	3,297.61	<0.0001
Preservative × storage temperature	2	1,883.56	<0.0001
Milk age within interaction preservative × storage temperature	70	99.34	<0.0001

¹IBC = individual bacterial count.

preservative; $A_{l(jk)}$ = the effect of milk age within the interaction storage temperature × preservative (storage times at 4°C: 0, 1, 2, 3, and 5 d for NP; and 0, 1, 2, 3, 5, 7, 9, 11, 14, 18, and 22 d for AZ and BR; storage times at 10°C: 1 and 2 d for NP and 1, 2, 3, 5, 7, 9, 11, 14, 18, and 22 d for AZ and BR; storage times at -20°C of 1, 2, 3, 5, 7, 9, 11, 14, 18, and 22 d for NP, AZ and BR); and e_{ijkl} = the random residual effect.

For each analytical condition, the standard deviation of repeatability (s_r and its relative value, $s_r\%$, where $s_r\% = s_r \times 100/\bar{x}$, and $\bar{x}$ is the arithmetic mean of IBC) was calculated according to IDF standard 128A (IDF, 1999) using the formula

$$s_r = \left(\frac{1}{2q} \sum_{i=1}^q w_i^2 \right)^{1/2},$$

where q is the number of samples and w_i is the absolute difference between duplicate IBC determinations.

The factors bulk tank milk sample, preservative, storage temperature, interaction preservative × storage temperature, and milk age within the interaction preservative × storage temperature contributed significantly to the observed logIBC/mL (Table 2). Preservation strategy was the main factor that affected the logIBC/mL in goat milk samples. As expected, the highest ($P < 0.001$) IBC were obtained in the milk samples free of preservatives (7.312 ± 0.006 logIBC/mL). In preserved samples, the highest ($P < 0.001$) IBC were obtained for the AZ treatment (6.561 ± 0.004 logIBC/mL), and the lowest ($P < 0.001$) IBC were in samples preserved with BR (6.296 ± 0.004 logIBC/mL). Bronopol is a bactericidal preservative with a broad spectrum of antibacterial activity that causes membrane damage and could render the membrane impermeable (Stretton and Manson, 1973), possibly explaining the differences in IBC observed here between preserved samples.

The least squares mean of the logIBC/mL was greater ($P < 0.001$) at 10°C (7.012 ± 0.006) than at 4°C (6.690 ± 0.004) or -20°C (6.467 ± 0.004). Moreover, effects of the interaction preservation treatment × storage temperature revealed that the change in the IBC was modified by the preservation strategy in the stored samples,

with the exception of BR-preserved samples stored at 10°C and at -20°C (Table 3). When we examined the effect of milk age within the interaction preservative × storage temperature, it was possible to observe the changes experienced by IBC under different test conditions. Hence, at zero hours, no significant differences were obtained between the IBC values in AZ, BR, or NP samples stored at 4°C. Azidol has been proposed as the best preservative for TBC determinations using the FC method in cow milk (Suhren and Walte, 2000). The maximum concentration of sodium azide is fixed by the IDF at 0.024 g/100 mL for SCC determinations (IDF, 2006), and at 0.03 g/100 mL for the determination of fats, proteins, and lactose (IDF, 2000). Elizondo et al. (2007) assessed the efficacy of different concentrations of sodium azide in AZ preservative when used for the preservation of sheep milk, and noted that a concentration of 0.006 g of sodium azide/100 mL allowed for more precise evaluation of the bacteriological quality of raw milk using the Bactoscan 8000 (Foss Electric). Consistent with these observations, we used the least squares mean of the IBC values obtained at zero hours in the AZ-preserved samples stored at 4°C as a reference value (6.527 ± 0.020 logIBC/mL, geometric mean = $3,365 \times 10^3$ IBC/mL). Accordingly, the least squares mean of the logIBC/mL recorded for the AZ-preserved samples stored at 4°C did not vary significantly from 0 to 11 d postcollection (Table 4). These observations are consistent with results obtained in cow milk stored at 4°C, in which the TBC of samples preserved with AZ at a concentration of 0.012 g of sodium azide/100 mL remained unchanged up to 15 d postcollection (Barcina et al., 1987). When the least squares mean of the logIBC/mL from AZ-preserved samples stored at 10°C was compared with values taken as reference (IBC recorded at 0 h in samples preserved with AZ stored at 4°C), the trend was irregular during the study period, and the highest values ($P < 0.01$) were obtained at 2, 5, 11, and more days postcollection. These results are in line with those obtained in sheep milk refrigerated at $6 \pm 2^\circ\text{C}$, in which preserving samples with AZ at the same concentration used in our study allowed for TBC determination over 3 d (Elizondo et al., 2007). When the least squares means of the logIBC/mL of

Table 3. Least squares means and standard errors of logIBC/mL¹ by storage temperature × preservative treatment

Storage temperature, °C	Preservative	LogIBC/mL	SE	IBC ²
4	Azidiol	6.488 ^a	0.006	3,076
	Bronopol	6.224 ^b	0.006	1,675
	None	7.358 ^c	0.009	22,803
10	Azidiol	6.736 ^d	0.006	5,445
	Bronopol	6.339 ^e	0.006	2,182
	None	7.962 ^f	0.014	91,622
−20	Azidiol	6.458 ^g	0.006	2,871
	Bronopol	6.325 ^e	0.006	2,113
	None	6.617 ^h	0.006	4,139

^{a-h}Means within a column with different superscripts differ significantly ($P < 0.001$).

¹IBC = individual bacterial count.

²Geometric mean ($\times 10^3$ IBC/mL).

AZ-preserved samples stored at −20°C were compared with the reference values, the lowest values ($P < 0.01$) were obtained from 7 to 22 d postcollection. Therefore, according to the accuracy, freezing at −20°C did not add any additional benefit to the milk preserved with AZ.

Bronopol-preserved samples stored at 4 and 10°C for 24 h showed lower ($P < 0.001$) logIBC/mL values (6.343 ± 0.0199 and 6.312 ± 0.0199 , respectively) compared with the AZ preserved samples at time zero. The reduction ($P < 0.001$) of the IBC obtained in BR-preserved and frozen samples at 24 h (6.315 ± 0.0199 logIBC/mL) remained stable over the study period and invalidated these analytical conditions for TBC analyses. The preservation of goat milk samples with BR allows for the determination of milk constituents and SCC over long periods of storage (Sánchez et al., 2005). However, because of the different IBC obtained for the BR-preserved samples, we recommend that this pre-

servative should not be used in goat milk samples for TBC determination using the automated FC method. For practical purposes, the automatic determination of SCC, composition, and TBC in the same milk sample should be performed using refrigerated and AZ-preserved samples.

The NP samples stored at 4 or 10°C showed significantly higher logIBC/mL at 24 h postcollection (7.125 ± 0.0199 and 7.582 ± 0.0199 , respectively). Our results are consistent with those obtained in cow milk, in which markedly increased TBC have been observed in NP samples stored at 0 to 2°C (Suhren and Walte, 2000). Frozen NP samples showed a significant increase in IBC at 24 h (6.665 ± 0.0199 logIBC/mL), which remained constant during the study. This could be explained by the fact that our milk aliquots were kept in heat-insulating polyethylene tubes, possibly inducing slow freezing such that bacterial growth could take place. The increased IBC recorded in the NP samples

Table 4. Least squares means of log individual bacterial count (IBC)/mL, geometric means (GM, $\times 10^3$ IBC/mL), and standard deviations of the repeatability (s_r and $s_r\%$) of IBC determined by the BactoScan flow cytometry method calculated from log-transformed (s_r) and untransformed values (s_r and $s_r\%$) over the study period according to the storage temperature (4, 10, and −20°C) for goat milk samples preserved with azidiol

Days post collection	4°C					10°C					−20°C				
	LSM ¹	GM	s_r , log units	s_r , IBC/mL	$s_r\%$	LSM ¹	GM	s_r , log units	s_r , IBC/mL	$s_r\%$	LSM ¹	GM	s_r , log units	s_r , IBC/mL	$s_r\%$
0	6.527 ^a	3,365	0.014	173	3.10	—	—	—	—	—	—	—	—	—	—
1	6.560 ^a	3,631	0.015	229	3.69	6.579 ^a	3,793	0.011	79	1.22	6.483 ^a	3,041	0.015	112	2.11
2	6.541 ^a	3,475	0.012	97	1.63	6.615 ^a	4,130	0.011	124	1.76	6.476 ^a	2,992	0.011	160	3.06
3	6.544 ^a	3,499	0.035	129	2.14	6.577 ^a	3,776	0.008	120	1.82	6.463 ^a	2,904	0.014	169	3.30
5	6.526 ^a	3,357	0.011	133	2.32	6.609 ^a	4,064	0.015	128	1.81	6.471 ^a	2,958	0.012	162	3.10
7	6.488 ^{ab}	3,076	0.017	107	2.02	6.587 ^a	3,864	0.011	136	1.91	6.446 ^a	2,793	0.017	234	4.73
9	6.473 ^{ab}	2,972	0.029	117	2.27	6.587 ^a	3,864	0.015	208	3.04	6.455 ^a	2,851	0.015	133	2.64
11	6.462 ^{ab}	2,897	0.015	187	3.72	6.630 ^a	4,266	0.014	217	2.84	6.444 ^a	2,780	0.016	147	3.00
14	6.410 ^b	2,570	0.017	149	3.25	6.809 ^b	6,442	0.024	472	4.87	6.451 ^a	2,825	0.016	140	2.83
18	6.426 ^b	2,667	0.021	154	3.24	6.917 ^c	8,260	0.010	282	2.36	6.450 ^a	2,818	0.014	179	3.61
22	6.411 ^b	2,576	0.047	343	7.34	7.448 ^d	28,054	0.017	875	2.85	6.446 ^a	2,793	0.018	224	4.61

^{a-d}Means within a column with different superscripts differ significantly ($P < 0.01$).

¹SEM of LSM = 0.0199.

precludes their use for TBC at all the storage temperatures examined here.

Table 4 shows the s_r values calculated from the log-transformed (s_r) and nontransformed (s_r and $s_r\%$) variable in our IBC repeatability study for all the study times in AZ-preserved samples. In all the preservation strategies examined, s_r (log units) were similar to those reported by other authors for raw cow milk (Ninane et al., 2000; Suhren and Walte, 2000; Bolzoni et al., 2001) and sheep milk (Tomáška et al., 2006) and always <0.05 , which is the mean value of the BactoScan FC (Foss Electric) provided by the manufacturer. The mean s_r recorded were 0.015, 0.021, and 0.014 for the NP, AZ-preserved, and BR-preserved milk samples, respectively, refrigerated at 4°C; 0.016, 0.014, and 0.018, respectively, for samples refrigerated at 10°C; and 0.015, 0.015, and 0.026, respectively, for samples frozen at -20°C.

Absolute s_r values were proportional to the mean IBC recorded for all the ages of milk except for the frozen samples preserved with BR, which showed higher s_r than frozen samples preserved with AZ. As in the case of the s_r (log units), relative values of the standard deviations of repeatability ($s_r\%$) were similar for both the preserved and nonpreserved milk samples. Accordingly, the s_r (log units) and $s_r\%$ recorded here for the IBC were adequate, indicating that the BactoScan FC measurements were reliably repeatable for goat milk samples stored under the analytical conditions assessed.

In conclusion, the use of samples preserved with AZ stored at 10 or 4°C are appropriate for TBC determinations by the BactoScan FC method for up to 24 h and 11 d postcollection, respectively.

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