PAPER

NEW NATIONAL CONVERSION LINE FOR BACTOSCAN FC IN ITALY: A STEP FORWARD

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ABSTRACT

To improve the reproducibility of flow cytometry technique for total bacterial count in milk, a conversion from instrumental results (impulses/μL) to the reference method resultes (cfu/mL) is needed. In 2008 in Italy, a project for a common conversion line for Bactoscan FC was initiated. In this paper we report on the second phase of the project focusing on the statistical procedure used to evaluate the validity of the data. The new conversion line, representative of national milk (2,732 valid samples from 29 labs) obtained from both rounds of the study is: Log_{10} (cfu mL^{-1}) = Log_{10} (IBC μL^{-1}) x 0.939 + 2.559, with $S_{y:x}$ = 0.282 with an application range up to 70,000 IBC μL^{-1}.

- Keywords: Bactoscan FC, conversion line, cow milk, total bacterial count -

INTRODUCTION

Regulation (EC) 1664 (EC 1664:2006) established that the reference method for determining total bacterial count at 30°C in raw milk is EN ISO 4833 (ISO 4833:2003), however the use of alternative methods is acceptable when they are validated against the reference method in accordance with the protocol set out in EN/ISO standard 16140 (ISO 16140:2003) or other similar internationally-accepted protocols. In the case of milk, ISO 21187 (ISO 21187:2004) and ISO 16297 (ISO 16297:2013) are examples of other such protocols.

EN ISO 4833 instructs that colonies grown in defined conditions must be counted after 72 h of incubation at 30°C whereas flow cytometry instruments count free cells independently from their physiological status or their capability to develop into a colony. The counts are obtained from electrical impulses (derived by the fluorescence of bacterial DNA and RNA stained by fluorochrome ethidium bromide) and must be converted into cfu mL^{-1} equivalents, as this is the regulatory unit of measure. This conversion (when calculated by a single laboratory) is the main reason for the low reproducibility of the alternative method in spite of its otherwise better repeatability, rapidity and cost effectiveness compared to the reference method and this could have major consequences both from economic and food safety points of view. Currently the flow-cell automatic instruments for total bacterial count are indispensable to the centralized and specialized laboratories in charge of large numbers of milk samples per day. For this reason, at the end of 2008, the Reference Centre for Bovine Milk Quality of IZSLER launched a project for a "common conversion line" for Bactoscan FC (Foss, DK), the most commonly used instrument in Italy. The result of that study (BOLZONI and MARCOLINI, 2010) was adopted on a voluntary basis by several laboratories in our country in the last few years. In 2012, with the coordination of the Italian National Reference Laboratory (NRL) for milk (Istituto Superiore di Sanità), a second round of the project was developed with the objectives to: verify the results of the first round of the project; study a wider range of milk contamination levels; derive a conversion formula that is more tailored to Italian milk, meaning a single, mandatory conversion formula to be applied at the national level; propose a statistical model to evaluate the reliability of the raw data.

MATERIALS AND METHODS

The study involved 29 laboratories from all over Italy. The number of samples analyzed from each laboratory and for the different levels of contamination was determined on the basis of their previous participation or not in the first round of the project in 2008 (Table 1).

The protocol adopted in 2008 (BOLZONI and MARCOLINI, 2010) was adopted again in 2012 with the intention of producing comparable data. Participating laboratories, during the period from January to June 2012, selected samples of cow bulk tank milk (refrigerated and without preservatives) from those submitted for daily analytical activity. The instrument's calibration status was checked through an inter-laboratory trial using lyophilized milk samples at 3 different contamination levels that were shipped to participants (data not shown). Considering that ISO/TS 19036 (ISO 19036, 2006) estimates that the standard deviation for aerobic mesophilic flora in milk (S_R = 0.12) is affected more by operative conditions (S_{cond} = 0.09) than by the initial suspension (S_{IS} = 0.04), it was decided that the reference method would be performed using 2 plates per dilution with one series of dilutions. In each laboratory, immediately before analysis, each sample was mixed as stated in ISO 6887-5 (ISO 6887-5, 2010), tested in duplicate by the Bactoscan FC and immediately analyzed by the reference method. A single series of at least 3 decimal dilutions was pre-

Table 1 - Selection of samples – percentage of samples and respective ranges of impulses required from each lab.

Range Impulses (IBC µL-1)	% samples analyzed (from 10 to 50 samples)[A]	% samples analyzed (from 50 to 100 samples)[B]
0-20	3	3
21-100	30	10
100-1,000	30	10
1,000-5,000	25	10
5,000-10,000	4	30
10,000-50,000	4	27
50,000-99,999	4	10

A: Laboratories WITH participation in the project prior to 2009.
B: Laboratories WITHOUT participation in the project prior to 2009.

pared with quarter-strength Ringer's solution (the level of dilution was established on the basis of the previous instrumental results); 1 mL of each dilution was dispensed in each of 2 plates of milk-PCA medium and then incubated at 30°C ± 1°C for 3 days. Each participating lab contributed their data on the Bactoscan FC double counts in "impulses" (IBC μL^{-1}) and colonies counts from the two plates of each dilution to a database. After the relevant controls of raw data (see: point "d" in the "selection of results" section below) as indicated by ISO 7218 (ISO 7218:2010; ISO 14461-2:2005) and the additional controls (see: points "e" and "f"), the linear mixed effect model (LME) was applied to produce the regression line of the data from the "valid samples". The statistical evaluation of the results is described in the following section. The software "Procedure R 2.15" and Excel 2010 (Microsoft Corp., Redmond, WA) were used.

RESULTS AND DISCUSSION

Range of measurement and linearity

The ratio between observed values (O.V.) and expected values (E.V.) in impulses μL^{-1} (IBC μL^{-1}) from serial dilutions of *ad hoc* heavily contaminated milk samples was taken as an indicator of linearity of the instrumental signal response. The ratio O.V./E.V. ~ 1 (Fig. 1) suggests the acceptable instrumental linearity continues up to 50,000 IBC μL^{-1}, which is well above the producer's declared limit of 30,000 IBC μL^{-1} and confirms our previous evaluation (BOLZONI *et al.* 2000, BOLZONI *et al.,* 2001).

Since one of the aims of the work was to evaluate whether a broader range of instrumental measures could be accepted without affecting the conversion line, values > 30,000 IBC μL^{-1} were also considered. Ratio O.V./E.V. = 0.9 was adopted as an arbitrary lower limit of acceptability of the linearity indicator (equivalent to 3 standard deviations from the mean of the ratios obtained). These considerations allowed us to accept 70,000 IBC μL^{-1} as the upper limit for the range of application of the conversion line. We would like to note that samples with IBC μl^{-1} > 30,000 (approximately > 4,000,000 cfu mL^{-1}) are rather unusual in Italy.

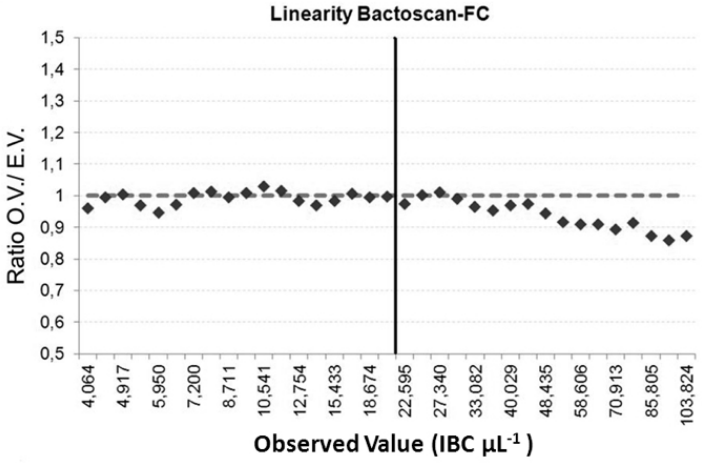

Fig. 1 - Bactoscan FC linearity: the relationship between the observed values and the ratio of the observed value to the expected value.

Selection of results

Of the 1,827 total milk samples analyzed, which is equivalent to more than 10,000 analytical results produced by 29 participating laboratories, the selection process for valid data led to the rejection of 499 (27%) samples due to the following factors:

a) *Unreliability* – 19 samples were eliminated for absence of correspondence between the instrumental results and the reference method results or errors in the report transmission results.

b) *Out of range of measurement* – 65 samples were eliminated because their values were outside the established range of linearity (12 samples lower than 10 IBC μL^{-1} l and 53 higher than 70,000 IBC μL^{-1}).

c) *Instrumental repeatability* – 31 samples were eliminated because the difference between replicates exceeded the repeatability limit of the Bactoscan FC: Critical Log Difference between replicates > 2.83 S_r (P 95%). Additionally 12 samples were eliminated because they exceeded the instrumental reproducibility limit (S_R).

d) *Maximum - minimum numbers of colonies on the plates and proportionality between dilutions* – plates outside the range 10 - 324 colonies were not considered for the count (ISO 7218:2007). The G^2 factor test, which compares the relationship between pairs of plates and dilutions, led to the elimination of 179 samples.

e) *Sub-dispersion of reference method results* - no laboratories were eliminated on this basis (which compares the relationship between observed and expected values on plates) but the frequency of sub-dispersed samples was one criterion used for the selection of laboratories described in point f.

f) *Single laboratory performance evaluation* – the effect of each individual laboratory on the extrapolation of the final regression line was considered on the basis of the following factors:

- excessive or insufficient dispersion of the individual lab's regression line;
- high frequency of sub-dispersed results from the reference method;
- high frequency of eliminated results from the G^2 factor test.

The dispersion of data around single-lab regression lines is reported in Table 2 as $S_{y:x}$. Giv-

Table 2 - Dispersion of the conversion line for individual laboratories (S y:x).

Lab Code	Samples (n)	Intercept	Slope	Sy:x
40	50	2.1184	1.0309	0.0139
27	98	2.9025	0.7797	0.0930
14	42	2.4432	0.9911	0.1455
38	36	2.2563	1.0279	0.1577
31	93	2.3363	1.0408	0.2517
35	52	2.1976	1.0711	0.2556
41	16	2.1718	1.0859	0.2594
1	40	2.1280	0.9966	0.2676
39	88	2.6219	0.8914	0.2766
15	26	2.5538	1.0119	0.3086
11	26	2.5408	0.9711	0.3118
23	98	2.6394	0.9257	0.3223
24	50	2.4829	0.9593	0.3291
6	68	2.2774	1.0927	0.3365
28	54	3.5260	0.6413	0.3546
37	79	3.6620	0.5508	0.3707
22	76	2.7561	0.8592	0.3756
26	55	2.1806	0.9484	0.3766
7	22	2.4747	1.0033	0.3830
29	24	2.7238	0.9293	0.3893
33	89	3.0733	0.6950	0.4104
34	103	2.1782	1.2029	0.4145
25	97	2.8959	0.7690	0.4286
30	36	2.8759	0.7964	0.4379
8	34	2.6250	0.9531	0.4410
32	29	3.1796	0.6974	0.4567
9	30	3.0099	0.8643	0.6225
36	32	2.5325	0.6897	0.6386
21	110	3.1939	0.8881	0.8504

en $S_{y:x} < 0.40$ is a criterion for acceptability (listed as a "tentative value" in ISO 16297:2013), nine of twenty-nine labs were over range. Of the nine, six were considered borderline and only laboratories 21, 36 and 9 were eliminated for over-dispersion. Furthermore laboratory 40 was eliminated for sub-dispersion, which suggested their results were not completely reliable. Two laboratories exhibited a high frequency of eliminated samples by the G^2 factor test (> 50% of samples); in the first case we decided to eliminate all results (Lab 36, which had already been eliminated for high dispersion as mentioned above), whereas in the second case (Lab 28) we decided to preserve the remaining "valid results" considering the very low value of dispersion of its regression line (0.3546 $S_{y:x}$).

Evaluation of the regression line

The LME model was applied to produce the regression line of the selected 1,388 valid samples. Multi-step selection of outliers (residual standard deviation > 2.58) was preliminarily applied (ISO 21187:2004). In synthesis, after a 3-step sequential elaboration, 65 outliers were eliminated, narrowing the number of valid results to 1,323 and improving the $S_{y:x}$ value from 0.3547 to 0.2781. After the third step, no significant improvement in the level of estimation could be obtained so no further elimination of data was considered appropriate.

The following conversion equation was calculated from the 1,323 residual samples (characteristics of the conversion equation are reported in Table 3):

$$\text{Log}_{10}(\text{cfu mL}^{-1}) = \text{Log}_{10}(\text{IBC } \mu\text{L}^{-1}) \times 0.946 + 2.569$$

Fig. 2 shows the conversion line from 2012 alongside the conversion line from 2009 (black dashed line) (6), calculated by:

$$\text{Log}_{10}(\text{cfu mL}^{-1}) = \text{Log}_{10}(\text{IBC } \mu\text{L}^{-1}) \times 0.911 + 2.599$$

The conversion line from 2012 is very similar to the line from 2009 although differences are seen at high and very high contamina-

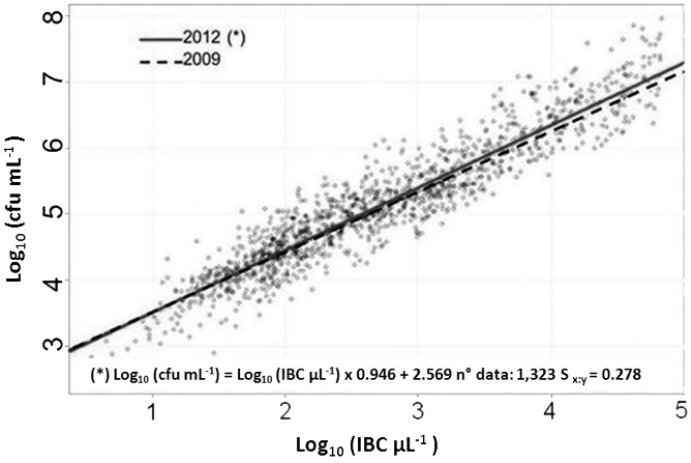

Fig. 2 - Distribution of data from the 2012 conversion line compared with the 2009 conversion line.

Table 3 - Characterization of the conversion line from 2012.

Parameters	Coefficient	St. error	T	Sig	Low	High
Intercept	2.569	0.038	67.57	0.000	2.493	2.645
Slope	0.946	0.009	106.91	0.000	0.928	0.964
Number of samples = 1,323; S y:x = 0.278.						

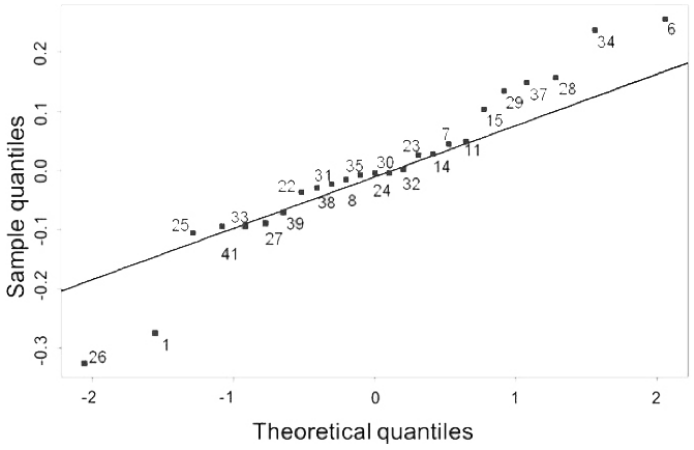

Fig. 3 - Q-Q plot of random effects from each laboratory in the Linear Mixed Effect Model

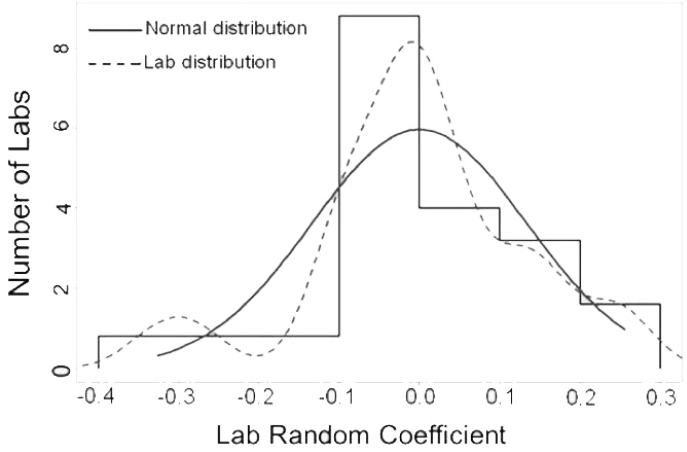

Fig. 4 - Distribution of random effect coefficients from the labs compared with a normal distribution (2012 conversion line).

tion levels as a consequence of the extension of the measurement field in the second round of the project.

In Figs. 3 and 4, the distribution of random effects in the LME for data from individual laboratories is presented. Statistically four labs were found to be apparently different from the others: numbers 6 and 34 overestimated their counts while numbers 1 and 26 underestimated their counts. No factors affecting this distribution could be identified (e.g. bacterial flora, sample characteristics, or systematic bias in reference method execution), so the data from these labs were kept in the regression line calculation.

New national conversion line

Considering that the same procedure and the same statistical evaluation were used in both rounds of the project, we considered it not only possible but also appropriate to pool the valid results from 2009 and 2012 and to run a new mixed statistical evaluation. Taking a step back before the respective outliers were excluded, a new multi-step selection was performed on the 1,474 valid results from 2009 combined with the 1,388 from 2012. The total elimination of 130 samples at the third step of selection led to no further increase in estimation (Table 4).

The final regression line was computed from 2,732 samples and it is represented by the equation:

$$\text{Log}_{10}(\text{cfu mL}^{-1}) = \text{Log}_{10}(\text{IBC } \mu\text{L}^{-1}) \times 0.939 + 2.559$$

The characteristics of the combined regression line are reported in Table 5 and Fig. 5.

Table 4 - Multi-step selection of outliers on 2009 and 2012 aggregated data.

Step No.	Samples (n)	S y:x	Intercept	Slope	Min Std Residual	Max Std Residual
1	2,862	0.3533	2.591	0.921	4.503	-5.798
2	2,793	0.3048	2.575	0.931	2.989	-3.103
3	2,752	0.2886	2.565	0.937	2.707	-2.691
4	2,732	0.2821	2.559	0.939	2.651	-2.645
5	2,724	0.2796	2.558	0.939	2.660	-2.597
6	2,718	0.2778	2.557	0.939	2.620	-2.590

Table 5 - Characterization of the new national conversion line (2009 and 2012 pooled results).

Parameters	Coefficient	St. error	T	Sig	Low	High
Intercept	2.559	0.032	80.77	0.000	2.496	2.622
Slope	0.939	0.006	150.38	0.000	0.927	0.952
Number of samples = 2,732; S y:x = 0.282						

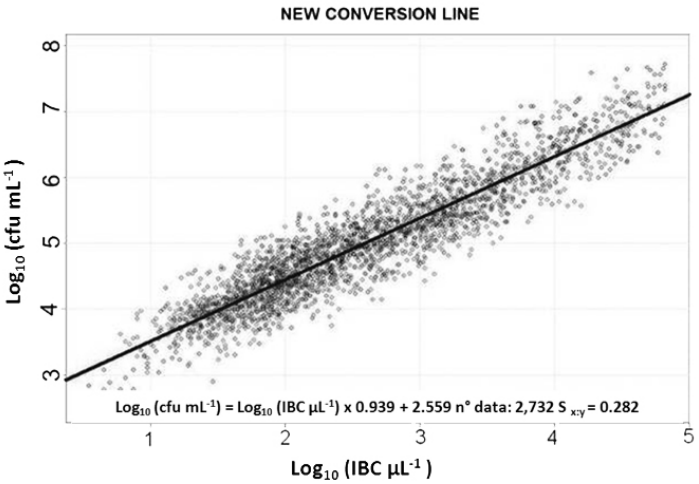

Fig. 5 - The new national conversion line (computed from 2009 and 2012 aggregated data).

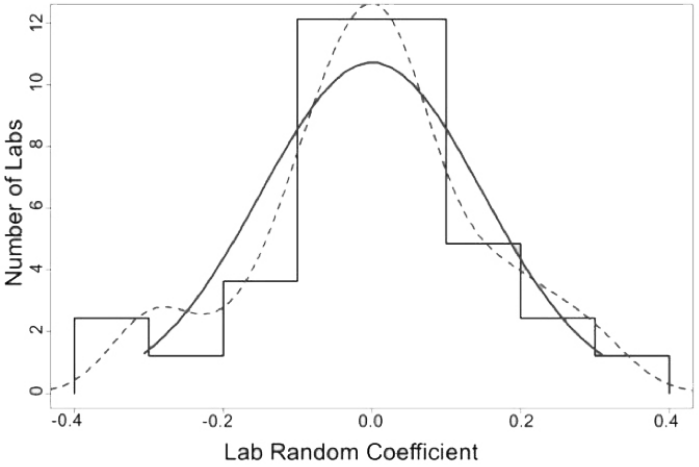

Fig. 6 - Distribution of random effect coefficients from labs compared with a normal distribution (new conversion line).

The distribution of random effects in the LME for individual laboratory data is presented in Fig. 6.

CONCLUSIONS

During the first round, in 2008, the main focus was on milk samples with total bacterial counts around 100,000 cfu mL^{-1}, the European Legal Limit for compliance (Reg. EC 853:2004). Contrastingly, during the second round, laboratories were invited to include milk samples with high to very high levels of bacterial contamination in order to test the instrumental response to bacteria levels outside of the linear range indicated by the Bactoscan FC producers. In routine situations the submission of very highly contaminated samples is a rare occurrence, however they should nonetheless be analyzed and their results should be entered into the geometric mean of the last three months, as per the calculation system defined by Reg. EC 853:2004.

The present project led to the creation of a conversion relationship between impulse μl^{-1} and cfu $mL^{-1}$for the enumeration of the total bacterial counts in Italian raw cow milk using a Bactoscan FC. In summary the conversion line incorporates the following points:

- the conversion relationship was constructed according to ISO 21187:2004;
- the level of accuracy obtained was satisfactory ($S_{y:x}$ = 0.282 $\log_{10}$);
- the number of samples was representative of Italian milk production variability;
- 80% of all Italian laboratories involved in milk control by routine method joined the project.

The new conversion line appeared robust and representative of milk quality and variety in Italy, with a range of application up to 70,000 IBC μL^{-1}. It was ultimately validated and adopted as the national conversion line in Italy. This is an important advance for both the industry and public hygiene because the use of a unique conversion line should significantly improve the reproducibility of the bacterial count results obtained by Bactoscan FC in Italy. In addition, the use of the conversion line for highly-contaminated samples is a further contribution to improve analytical harmonization. Data quality control was focused on the evaluation of data entry quality and consequently the accuracy and robustness of the elaborated conversion line. This was done by checking the raw data (agreement between pairs of plates, and proportionality between successive dilutions).

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