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BactoScan FC: conversion system for results at the national level in Italy and reproducibility of total bacterial count testing four years after implementation

Giuseppe BOLZONI[1]*, Antonio MARCOLINI[1], Annunziata FONTANA[2], Laura MONACO[2], Anna Maria FERRINI[3]

Abstract

BactoScan and BactoCount are automated instruments for the determination of the total bacterial count (TBC) in milk through an alternative routine method. Results are given in impulses, but the TBC is officially expressed in colony-forming units per ml (CFU/ml), making a conversion system necessary in order to transfer results onto the official scale. In Italy, these instruments were introduced at the beginning of the 1980s, and today amount to more than 50 units. The initial huge number of conversion lines was gradually reduced over the years until 2012, when a single conversion relationship, developed by a joint NRCBMQ – NRLMMP project, was finally made available to Italian laboratories. In fact, it has been adopted by almost all the laboratories that routinely use these instruments. This article examines the results of about 50 proficiency tests (PTs) organised by the Italian Breeders Association (AIA) on a national scale in the period 2003-2016, for which laboratories were asked to provide results in impulses and in CFU, according to their own current conversion system. A retrospective statistical analysis of the results enabled us to assess the changes in the reproducibility of the results expressed in both units of measurement over time: that is, in impulses (mainly dependent on instrumental performance) and in CFU (also dependent on the conversion line used). In particular, we demonstrate the effect of applying the national conversion system developed via the 2008-2012 harmonisation project.

Keywords

- Milk
- Total bacterial count
- Reproducibility of findings

[1] IZSLER, National Reference Center for Bovine Milk Quality, Brescia, Italy

[2] AIA, Standard Milk Laboratory, Rome, Italy

[3] ISS, National Reference Laboratory for Milk and Milk Products, Rome, Italy

* Corresponding author : giuseppe.bolzoni@izsler.it

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Introduction

The availability of the fully automated flow cytometry instruments BactoScan (Foss) and BactoCount (Bentley) for the determination of the total bacterial count (TBC) in raw milk with an alternative method has led, in a few decades, to an impressive improvement in laboratory performances for this parameter. This improvement involves especially repeatability, standardisation, speed of response, and reduction of costs compared to the same indicators related to the reference method ISO 4833-1:2013, since the reference method is a pour plate colony count method characterised by a predominantly manual and subjective component.

In particular, the speed of response has made these instruments extremely valuable for milk control laboratories, reducing the analysis time from 3 days for the reference method to a few minutes, making this alternative method particularly suitable for the dynamic workflow of milk processing.

Unfortunately, the drawback of this instrumental method is that results are obtained in impulses (IBC), whereas the official limit for TBC reported in Regulation (EC) No 853/2004 is stated in colony-forming units/ml (CFU/ml). The need to express the results in CFU/ml requires us to "convert" the instrumental results. Over about thirty years, this apparently simple step has led to a considerable number of studies and investigations carried out by several researchers or, individually by laboratories equipped with these instruments.

The different conversions applied by laboratories have had a remarkable impact on the final reproducibility of the results obtained since we are dealing with high technology instruments, characterised fundamentally by excellent precision.

The number of Italian laboratories expert in raw milk control on large numbers of samples per day is traditionally high (from about 20 to over 40 in the last two decades) and this has increased the impact of using different conversions more than in other countries. These conversion methods included internal systems, approaches adopted from other laboratories, or acquired directly from instrument manufacturers.

In 2008, the Reference Centre for Bovine Milk Quality at the IZSLER launched a national project for the evaluation of a single conversion system with the collaboration of 15 Italian laboratories (Bolzoni and Marcolini, 2010a; 2010b). In the following years, the work was strengthened through the participation of the Italian NRL-MMP (ISS). In order to harmonise the control of TBC, 33 public and private laboratories throughout the country were involved, and in 2012, a single national conversion system for bovine milk controls specific to BactoScan FC was validated (Bolzoni *et al.*, 2015).

This conversion was gradually implemented throughout the country, and it can be estimated that almost all the laboratories that currently control TBC with BactoScan FC in Italy have adopted this conversion system.

This work aimed at assessing the improvement in reproducibility achieved over time through the gradual reduction in the number of different conversion systems in use, until the adoption of the single conversion line. The work examined the collection of results obtained during periodic proficiency testing for TBC in raw milk using flow cytometry instruments (4 rounds per year on average) organised and implemented by the Standard Milk Laboratory of the Italian Breeders Association (AIA) since 2003 across Italy. This comprehensive database provides a dynamic picture of deviations among laboratories, with reference both to the results expressed in impulses and those expressed in CFU, calculated after applying the current conversion system used by each of the laboratories at the time of the PTs.

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Thirty years of "conversion" in brief

Obviously, it is not a simple task to solve a 30-year technical-scientific issue. After all, if we were to ask the question of how each bacterium or mass of bacteria behaves in each milk sample to form a colony in the conditions of the pour plate method, we would have to conclude that we do not know the answer. Let us recall briefly the core of the problem: automated instruments are able to count – with very good precision – all kinds of viable bacteria present in each raw milk sample analysed. However, it is not possible to deduce *a priori*, and with the same accuracy, how many colonies the same bacteria would have developed if analysed with the reference method. Unfortunately, the variability of the relationship of the results obtained with these two count methods is very highly dependent on the type and amount of bacteria in the sample, as well as metabolic conditions and growth and multiplication requirements, but also non-biological factors such as the energy applied when mixing the sample before analysis.

Of course, it is possible to develop a mathematical function that represents the correlation between impulses and CFU obtained in the laboratory from a representative number of samples, so as to correctly extrapolate the same function to the entire milk population from which it is derived, by statistical inference. Nonetheless, we are well aware that from the practical point of view, this is merely a type of “compromise”.

Among the many factors that may affect the variability of this relation, it must be highlighted that part of the microorganisms of a milk sample cannot grow and form colonies in the specific conditions of time, temperature, medium and atmosphere of incubation of the reference method. In addition, it should not be overlooked that the "gold standard" – meaning the result obtained with the reference method – is characterised by performances for repeatability and reproducibility that are less favourable than those obtained with instrumental counting.

Thirty years of studies, hypotheses and discussions cannot be summarised here, but we can point out the significant milestones:

- Description of the characteristics of the opto-fluorimetric method (Grappin *et al.*, 1985);
- Log transformed values and conversion through multiple change points procedure (MCPP) (Kaereby, 1990);
- Criteria and conversion modes (Shuren *et al.*,1991);
- Appropriateness of linear conversion with respect to polynomial conversion (Dasen *et al.*, 1990);
- Production of conversion relationships by single laboratories on a geographical basis (various authors from the 1990s to date);
- Study of the impact of factors influencing the conversion relationship (season, time between milking and analysis, temperature, mixing mode of the sample, etc.) (various authors from the 1990s to date);
- Issue of relevant International Standards (the main ones include ISO 21187, 2004; ISO 16140, 2003 and ISO 16297, 2013).

Regarding Italy, the first studies date back to the beginning of the 1980s for BactoScan III and to the end of the 1980s for BactoScan 8000. Since then and until 2008, the laboratories

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involved worked independently and in very different ways. Starting in the early 2000s, the introduction on the market of BactoScan FC resulted in a progressive convergence to two basic conversions: Shuren *et al.*, 1998; Bolzoni *et al.*, 2000 and Bolzoni *et al.*, 2001. Starting in 2010, the conversion system developed in the first part of the above-mentioned Italian project was gradually adopted by the participating laboratories. At the end of 2012, the national conversion system, produced and validated in the second part of the project, finally became representative of the entire national territory and became a technical reference for Italian laboratories.

Materials and Methods

Since the end of the 1980s, the Standard Milk Laboratory of the Italian Breeders Association has been performing PTs concerning the main analytical parameters of milk. These activities, initially directed to the laboratories of the Regional Breeders Associations and aimed at managing instrument calibration, were gradually extended to the vast majority of public and private laboratories working in the field.

PTs dedicated to the total bacterial count with flow cytometric instruments were started around 2003 with approximately four rounds per year to date (each consisting of 4 samples with different levels of bacterial contamination). The number of laboratories has of course changed over the years, as well as the types of instruments used. However, on the whole, the available volume of results represents an invaluable source of information covering a 13-year period.

For this work, data from about 50 PTs were examined, corresponding to a total of more than 200 milk samples analysed in the period between October 2003 and September 2016, by a number of laboratories ranging from a minimum of 15 in 2003 to a maximum of 46 in 2012 (accounting for the average number of 34 participating laboratories over the entire period

FIGURE 1 / Example of a synthetic result report of the elaboration provided by the organizer - PT of September 2015, unit of measure: Log Impulses/µl and Log CFU/µL

RING TEST TOTAL BACTERIAL COUNT – SEPTEMBER 2015

Log Impulses*1000/ml - **Repeatability – Reproducibility- Outliers**

Sample	Valid laboratories	Mean	r	R	Sr	SR	RSDr	RSDR	RSDL
1	41	2.381	0.053	0.259	0.019	0.092	0.784	3.850	3.769
2	42	2.716	0.032	0.090	0.011	0.032	0.412	1.176	1.102
3	42	3.350	0.024	0.203	0.009	0.072	0.256	2.146	2.130
4	39	3.928	0.014	0.204	0.005	0.072	0.129	1.838	1.834

General means

	Mean	r	R	Sr	SR	RSDr	RSDR	RSDL
	3.094	0.034	0.199	0.012	0.070	0.395	2.252	2.209

RING TEST TOTAL BACTERIAL COUNT – SEPTEMBER 2015

Log CFU*1000/ml - **Repeatability – Reproducibility- Outliers**

Sample	Valid laboratories	Mean	r	R	Sr	SR	RSDr	RSDR	RSDL
1	41	1.786	0.052	0.278	0.018	0.098	1.023	5.495	5.399
2	42	2.095	0.030	0.127	0.010	0.045	0.499	2.137	2.078
3	42	2.688	0.024	0.273	0.008	0.096	0.312	3.588	3.574
4	39	3.224	0.017	0.202	0.006	0.071	0.186	2.216	2.208

General means

	Mean	r	R	Sr	SR	RSDr	RSDR	RSDL
	2.448	0.033	0.228	0.011	0.080	0.505	3.359	3.315

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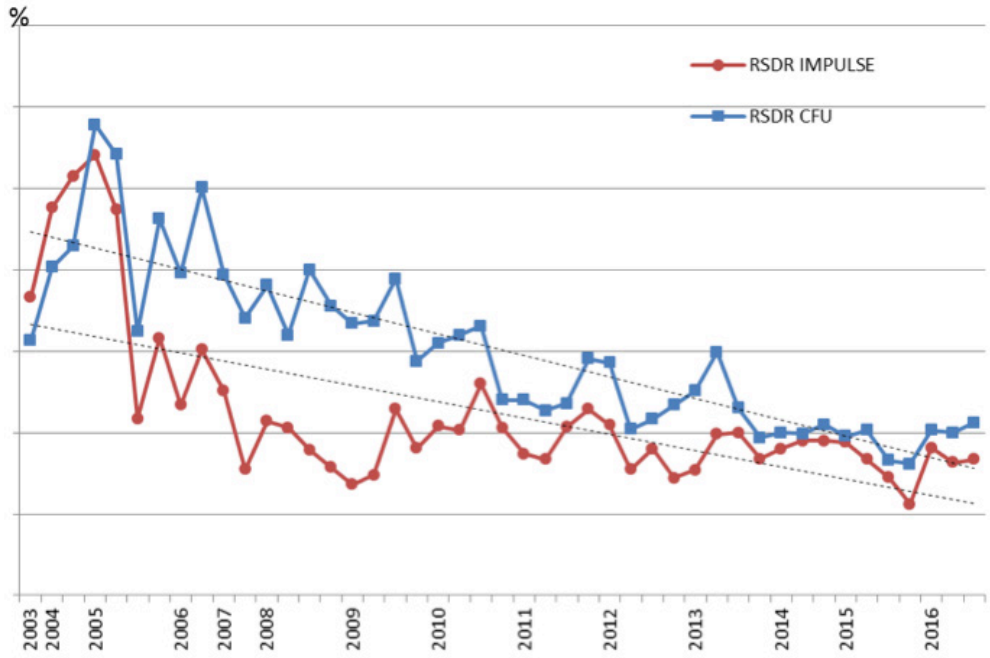
considered and 40 for the last six years). In the early years, the instruments available were BactoScan 8000 and BactoScan FC (initial ratio of 1:1), plus 1 BactoCount. The BactoScan 8000 model, which was used in 2006 in less than 10 laboratories, was gradually and completely replaced by the FC model by 2008. Moreover, as of 2008, two laboratories using BactoCount instruments also participated in the PTs.

The statistical analyses of the individual rounds have always been performed by AIA on the pooled data (with in-house software based on ISO 5725-2, 1994), without any distinction concerning the instruments used. Hence, no specific information is available for this study, but this is probably of little importance due to the disproportion in numbers consolidated in the last ten years.

The reports for each PT managed by the Standard Milk Laboratory of AIA provide, with reference to each sample and for each laboratory, the determination of the conventional indicators of dispersion and comparison with the reference values for the z-score evaluation for both units of measurement. The overall evaluation of each PT is also accompanied by graphics and tables for the two different units of measurement. An example of the summary page of a PT (September 2015) is provided in Figure 1, which shows the data in log impulses and log CFU for each sample with reference to: the number of valid laboratories (non-outlier labs), the average values, repeatability (r) and reproducibility (R) with standard deviations (Sr, SR) and finally, the estimated relative standard deviation of repeatability (RSDr), of reproducibility (RSDR) and of laboratory (RSDL); the same indicators are reported in the lower part as an overall estimate for the single PT.

The statistical analysis carried out in this study was performed using Excel software and taking into account the RSDR and RSDL values expressed in linear units and obtained from each PT or each sample, throughout the last 13 years. This unit of measurement was chosen for its consistency with the data available for the entire period, since the statistical evaluation on log transformed results was introduced by the PT organiser only recently.

FIGURE 2 / RSDR trend for impulses and CFU for PTs organized in the period 2003-2016



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Results and discussion

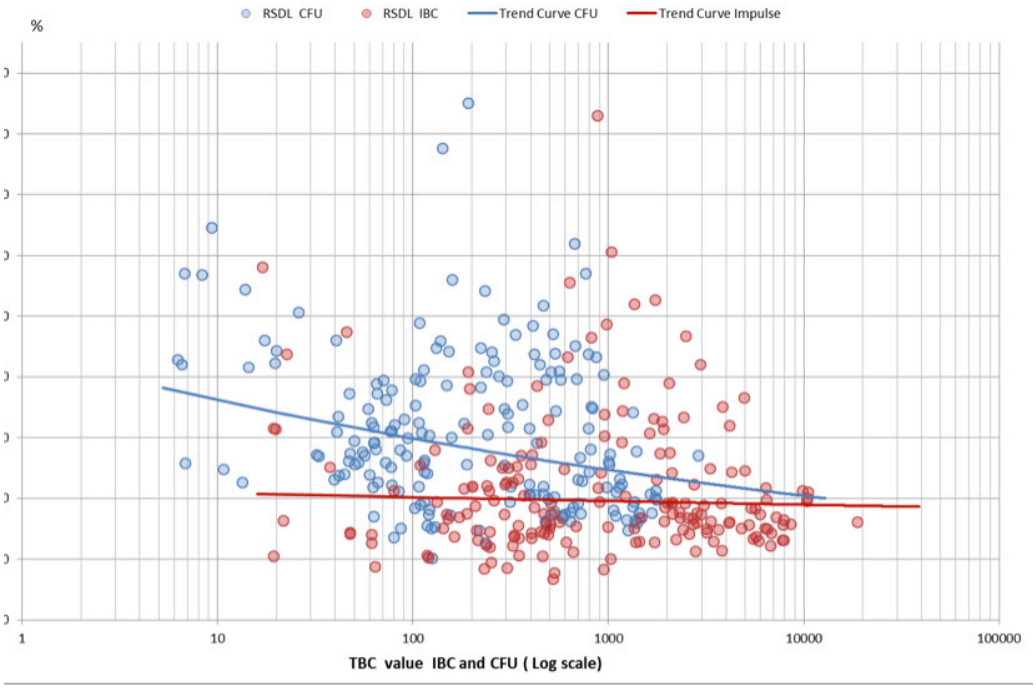
The first indicator chosen to estimate the trend of the "conversion effect" over time is the average RSDR value of each PT calculated on the valid results given both for the instrumental measurement unit (impulses) and after the conversion into CFU, as calculated by each participant. While the first results are instrument-dependent, the second ones are also dependent on the conversion mode in use in each laboratory, at the time of the PT.

Figure 2 shows the RSDR trend for the results given in both units of measurement. The values used for this graphic are those reported by the organiser as "general average of the PT" and show the linear trend estimated for the two series of values and their different slopes.

A more accurate evaluation can be obtained by replacing the indicator RSDR with RSDL, which represents the relative standard deviation among laboratories. For any given level, RSDR represents the overall reproducibility of the PT and incorporates both RSDr (independent of the different conversions applied by each laboratory during the PT) and RSDL (likely conditioned, among the other causes, by the variety of the conversion systems used).

Figure 3 shows the distribution of the RSDL values for the individual samples (given in impulses and in CFU). This specific distribution of values shows the influence of samples with very low bacterial contaminations on the overall evaluation, given the percent expression. This effect can be seen in the left part of the distribution, where the percentages for the individual samples appear to deviate significantly from the trend curve for CFU.

FIGURE 3 / Distribution and trend of RSDL values for individual samples (Pulses and CFU)



After removing 12 samples below 20 CFU/ml, a second analysis, similar to that plotted in Figure 2, was performed.

Figure 4 presents the RSDL values for the two units of measurement, calculated for each individual sample in the series of PTs considered. In the same figure, the results are reported both for the individual samples (shaded indicators) and for the averaged values for each sample group (solid indicators); the mobile media lines (solid lines), calculated as the rolling

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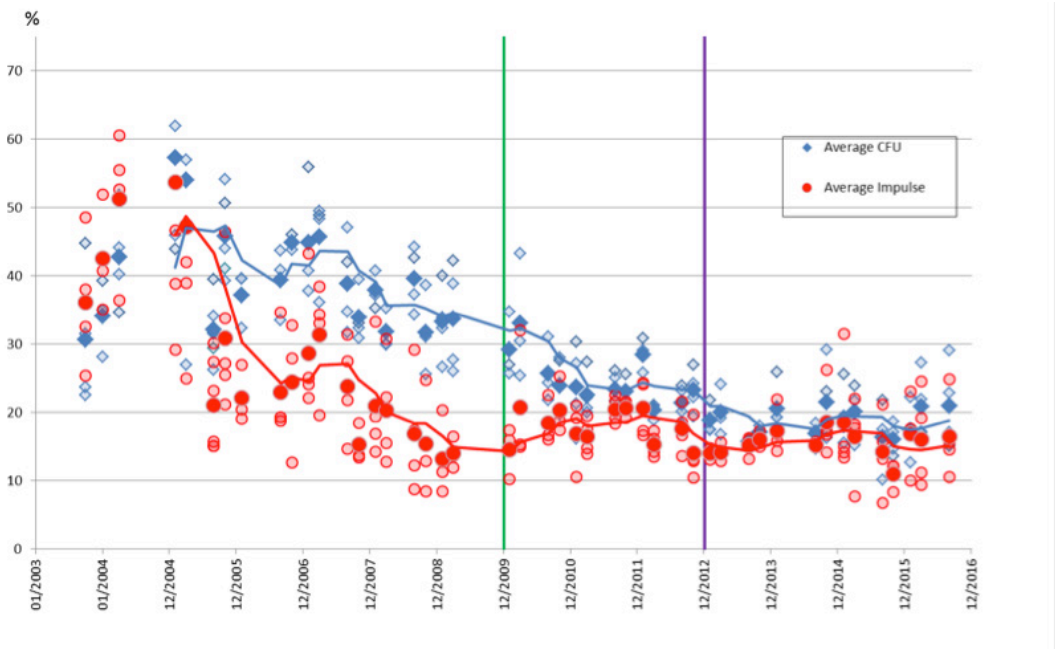
FIGURE 4/ RSDL values for impulses and CFU (for each sample and for averaged results); rolling averages and project phases



average of 4 consecutive values are also represented. Finally, the two vertical lines represent the temporal phases of the entire project.

The general trend shown in the graph is consistent with the previous results plotted in Figure 2. Moreover, it shows not only the gradual reduction of both the RSDL values over time, but also the clear tendency for the approachment of the two series of data. This enables us to draw the following conclusions:

Impulses - As an indicator of the performance of each instrument used by the participating laboratories, there is a clear improvement (reduction of the average RSDL from levels close to 50% for the first rounds to less than 20% for the most recent ones). This can be explained mainly by actions aimed at ongoing monitoring, maintenance or modification of instruments (for example autofocus, cleaning treatments of circuits, etc.) carried out after the rounds in the first period or implemented by outlier laboratories. In fact, the most significant improvement is concentrated in the rounds related to the first 2 or 3 years, and since 2009 substantial stabilisation to "physiological" values has been reached.

In addition, the gradual decommissioning of BactoScan 8000, completed in 2008 in favour of the new FC model, seems to support this observation.

Colony-forming unit - As an indicator of the conversion mode used by the participating laboratories, the decreasing RSDL values show a similar trend, and this must clearly be associated with greater uniformity of the results in impulses. However, as expected, the decrease appears to be delayed in time and in particular is more consistent than that of impulses: we believe that these two aspects are those that indirectly confirm the gradual effect of the conversion change adopted by the participating laboratories in the period considered. Although the milestones of this gradual process date back to 2010 and 2013 (first and second phase of the project), it is difficult to identify a net change in the plotted trends. The new conversion mode was in fact progressively implemented after the dissemination of the results of the two phases of the project. Moreover, some laboratories intentionally transitioned to the new conversion in 1-2 years with the aim of reducing the possible impact on the classic levels of TBC results they had been producing in their geographic area.

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The residual difference, albeit minimal and apparently constant, seems to persist even in the last rounds and could be accidental, given the very low level: the average RSDL of PTs from 2013 to 2016 is calculated as 20.6 for CFU and 16.5 for impulses, *i.e.* a minimal difference that theoretically should be destined to disappear. At present, the possible cause of this permanent difference could be the persistence of conversion modes other than the national system among the participants in the PTs. This applies, for example, to the two BactoCount systems currently in use, but probably also to a few laboratories in the dairy industries and private laboratories which, although participating in national rounds of PTs, still maintain their own conversion lines. Another possible source of this difference could be that values in CFU and in impulses are evaluated independently by the organiser of the PT. In this way, the selection of outliers could lead to the occasional exclusion of different laboratories in the two series of results.

Conclusions

A retrospective analysis of the results of about 50 collaborative rounds organised by the AIA in Italy in the last 15 years has provided evidence to assess the changes in laboratory performances for the determination of TBC in raw milk over time.

Information from the periodic rounds on TBC with flow cytometry instruments carried out since 2003 has highlighted that the overall reproducibility level of the alternative method has been markedly improved over time, in particular after the initiative undertaken between CRQLB and NRL-MMP to evaluate, define and transfer a single conversion line at the national level.

It is important to highlight that this type of instrument is essential in order to perform an efficient and timely control of raw milk and to allow the food business operator to implement the appropriate measures in time so as to correct the situation in case milk fails to meet the criteria stated for TBC. The many laboratories in Italy equipped with these instruments allow highly accurate, hygienic, continuous control of the raw milk produced every day by thousands of farms throughout the country, with acceptable costs and an exceptionally short time of analysis. The performances of the laboratories can be considered established and, in addition to showing that the level of reproducibility of TBC results for milk produced in Italy is highly satisfactory, demonstrate the uniformity of evaluation of this parameter throughout the country.

The type of work carried out and the results obtained could be taken as a reference in national contexts characterised by similar operating conditions, such as a high number of laboratories and farms, and climate and environmental differences on farms.

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