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MICROVAL VALIDATION REPORT

Extension MCS report

MicroVal Project 2021LR97 / 2023-108 (TC)

Extension MCS report of BactoCount IBC™ 3.0 for Total Bacterial Count (ISO 16297, ISO 21187, ISO 16140-2, EU-RL MMP criteria) and Somatic Cell Count (ISO 8196-3, ISO 13366-2, ISO 16140-2) <u>in raw goat milk</u>

MicroVal study number: 2021LR97 / 2023-108 (TC)

Device: BactoCount IBC™ 3.0

MicroVal Expert Laboratory: ACTALIA Cecalait

Manufacturer: Bentley Instruments

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SUMMARY

Bentley Instruments requested ACTALIA Cecalait to perform a MicroVal Validation of their BactoCount IBC 3.0 device for the total bacterial count (TBC) and somatic cell count (SCC) **in raw goat milk**.

The BactoCount IBC 3.0 can offer simultaneous real-time analysis of SCC and TBC in raw milk with flow cytometer, **but in the frame of this validation, SCC and TBC were tested separately**. Scope of the validation cover only the separate use of TBC and SCC.

The instrument is equipped with a second laser and two other detectors which were not used in this present validation study (dedicated for other applications).

The instrument has a speed of up to 200 samples / hour (presence of a rack sampler) and is piloted through its specific software Nexgen, version N° 2.21.

This instrument is MicroVal validated for enumeration of somatic cells and total bacterial count in raw cow milk (**2021LR97**).

The present extension study was built according to ISO 16297 (1), ISO 21187 (2) and ISO 16140-2 (3) for TBC and to ISO 8196-3 (4), ISO 13366-2 (5) and ISO 16140-2 (3) for SCC.

Results obtained for TBC were also compared with the limits defined in the EU-RL document (8).

The programs used for the analyze of sheep and goat milks are the same than for cow milk (ratio milk / reagent, type of reagent, incubation time and protocol). With the agreement of the MicroVal technical board, the evaluation of preliminary parameters (Carry-over effect, linearity, limit of quantification, stability) was not performed again because those were already validated for raw cow milk. Only the repeatability and the accuracy were evaluated for TBC and SCC in raw goat milk.

Microval Working Group agreed that an interlaboratory study was in this case not required as the study concerns an extension of the already fully validated and certified method for cow milk. Moreover, it will be difficult to find enough laboratories for a meaningful interlaboratory study.

Impact of milk composition on the TBC was evaluated with former instruments for TBC with the same principle than the BactoCount IBC^{TM} 3.0. The evaluated instrument was the BactoCount^{TM} (ISO 16140 validated) and impact of the matrix on TBC was compared to impact observed with the BactoScan in 2006 (study conducted by AGROSCOPE)(7). For the BactoCount, the correlation coefficients observed with milk composition were low. The same observation was done with the BactoScan.

Additional data of interference effect of SCC on TBC are presented into this report (§ 2.4)

The BactoCount IBC^{TM} 3.0 was not yet validated for sheep and goat milk by an independent laboratory. **This study is the first official evaluation performed by an ISO 17025 accredited independent laboratory**. However, reagents used with this device are the same than those used with BactoCount IBC^{TM} already certified ISO 16140 certificate N°2013 LR 44.

The BactoCount IBC^{TM} 3.0 was commercialized in the first time in 2019.

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1. Introduction

The BactoCount IBC 3.0 is a fully automatic instrument that uses flow cytometry for the rapid, accurate and highly reliable enumeration of individual bacteria and somatic cells in raw milk. It was developed by the BENTLEY INSTRUMENTS, Inc company (US) and distributed in Western Europe by Bentley Instruments SARL (www.bentleyinstruments.eu).

These enumerations can be performed combined or individually. **In the frame of this validation, SCC and TBC were tested separately**.

1.1. Principle of the alternative method

The **BactoCount IBC 3.0** is a (fully) **automated flow cytometer** for the rapid enumeration of individual bacteria and somatic cells in raw milk. **The alternative method protocol** is based on **flow cytometry principle**, where the DNA contents in cells (somatic cells or bacteria) are stained with a fluorescent marker, then detected through fluorescence signal. This signal is finally converted into universal unit thanks to the Bentley's software, NexGen.

The instrument has a speed of up to 200 samples / hour.

The raw milk is sampled and dispensed into individual wells located on a carousel with temperature regulated at 50°C. There, the raw milk sample **is mixed with an incubation reagent**.

For TBC, the reagent serves to lyse the somatic cells, to solubilize the fat globules and proteins, to permeabilize the bacterial cell walls and **to stain their DNA**. The fluorescent marker intercalates rapidly with the bacterial DNA. The mixture is then sonicated twice during the incubation. The sonication process promotes the chemical breakdown of the interfering particles and disrupts the remaining bacteria colonies to improve the detection of individual bacteria (IBC) and reduce the background fluorescence. The cell debris, devoid of nucleic acid, become excluded from the analysis.

After the incubation, the mixture is transferred automatically to the **flow cytometer** where the bacteria are aligned and exposed to an intense laser beam which causes them to fluoresce. The fluorescence signal is collected by the optics, filtered, and detected with a photo multiplier. The fluorescence pulses intensity and height are recorded and used as gating parameters. The sorted pulses are then translated into individual bacteria count (IBC) and converted to CFU/mL (reference scale) after applying a conversion equation on the software NexGen. An "universal" conversion equation developed on a large database of samples representative of all potential sources of variation in the milk flora (according to ISO 21187|IDF 196) can be installed on the instrument as a startup conversion equation. For this study, the threshold was fixed at 0.4.

For SCC. The reagent clarifies the milk sample and permeabilizes the somatic cells and stains their DNA with fluorescent marker. The somatic cells fluorescence pulses intensity and height are recorded and used as a gating parameter. The sorted pulses are then translated into Somatic Cells Count (SCC) after calibration against a set of SCC reference samples on the software NexGen. There is no sonication step for SCC.

1.2. Scope

Raw goat milk

1.3. Restriction of use

None

1.4. Reference methods

For accuracy testing, the results obtained with the alternative method were compared to the results obtained with the reference method or with a relevant Bentley's device already validated:

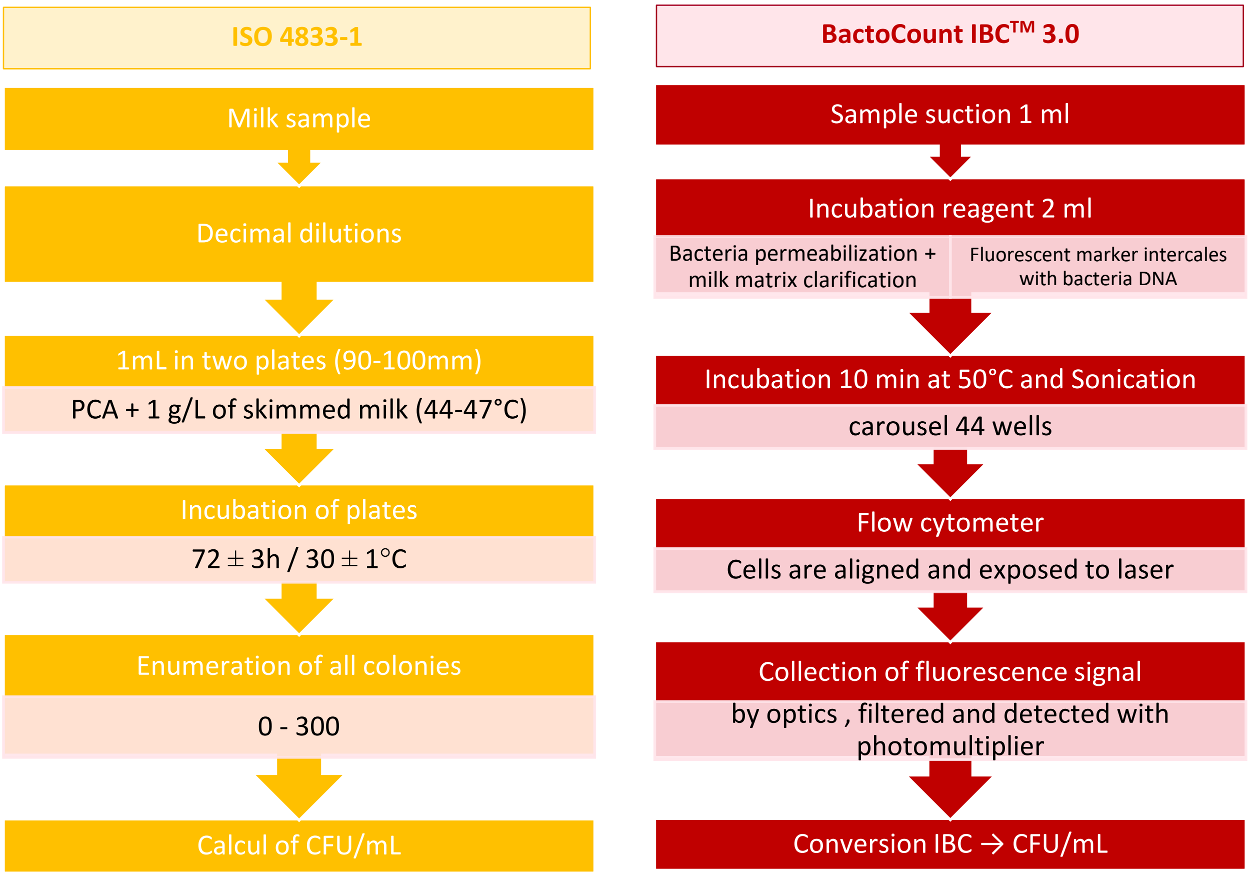
- For TBC: Reference method ISO 4833-1:2013+Amd 1:2022 (6);
- For SCC: SomaCount^{TM} FCM (ICAR certified for SCC in raw goat milk).

1.5. Conversion equation (IBC/CFU)

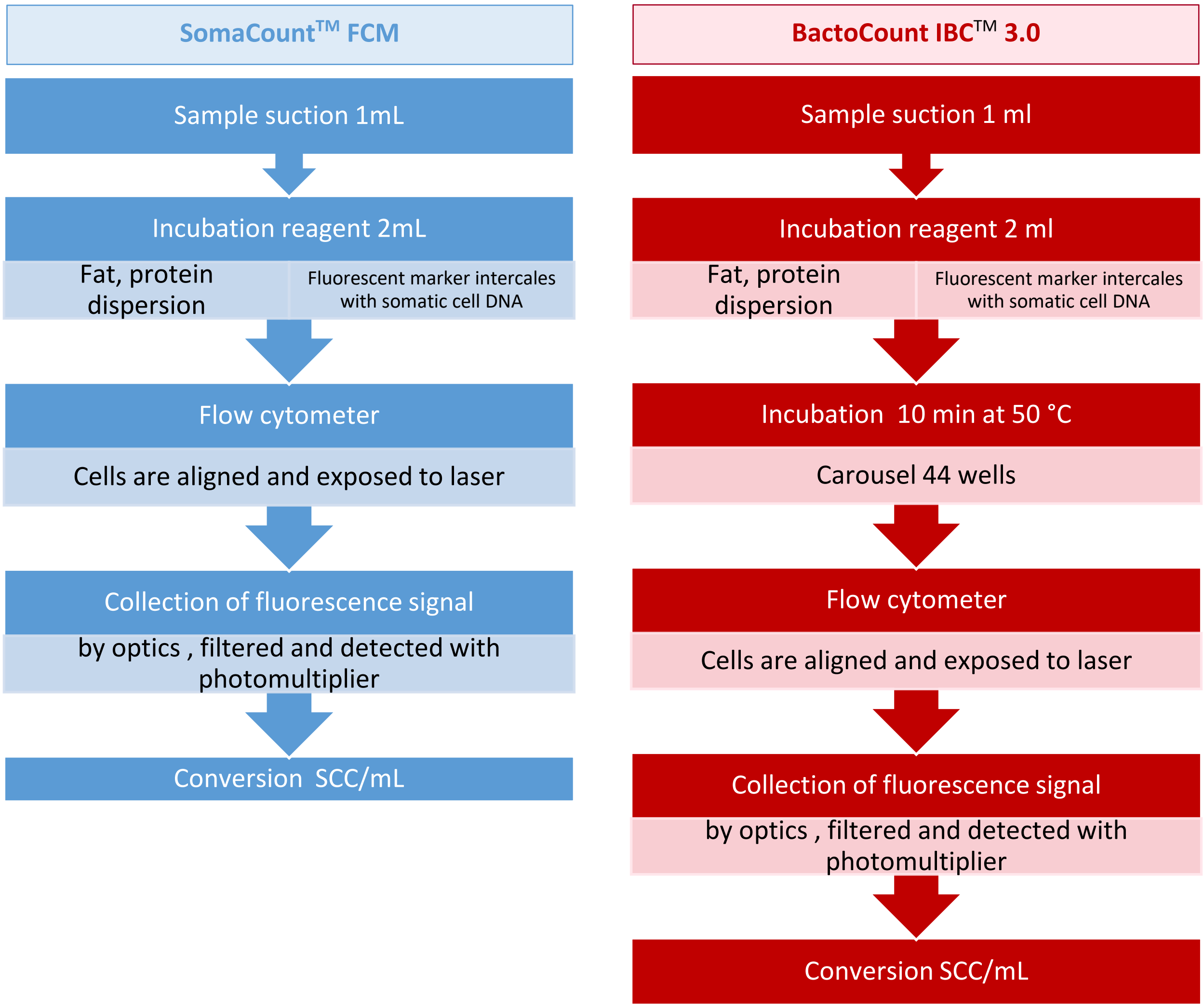
The device to be tested was equipped with a Universal Conversion Equation provided by Bentley Instruments (N° BactoCount U-CE 2013).

1.6. Validation procedure

The procedure for the direct comparison of **ISO 4833** method and **BactoCount IBC^{TM} 3.0** for **TBC** is schematically presented below.



The measurement procedure for the direct comparison of **BactoCount IBC™ 3.0** and the **SomaCount™ FCM** for the **SCC** is schematically presented below.



1.7. Safety precautions

Good Laboratory Practices for running food analyses were followed.

Extension of METHOD COMPARISON STUDY

FOR ENUMERATION OF

TOTAL BACTERIA (TBC)

In raw goat milk

2. Extension of method comparison study for enumeration of Total bacteria (TBC)

2.1. Materials and equipment used

- Goat herd milk samples;
- Stock and working solutions for BactoCount IBC™ 3.0, prepared according to manufacturer's instructions;
- RBS 2% solution (PN 9008) ;
- IBC incubation / dye solution (PN 3774G-KIT);
- IBC standard solutions (PN 9056);
- IBC microsphere solution (PN 3724F-900)
- Refrigerator at 0-4°C;
- Milk Plate Count Agar ;
- Petri dishes ;
- 1mL automatic pipette ;
- Water bath 44-47°C ;
- Tryptone-salt solution ;
- Incubator at 30 ±1°C ;
- Others standard laboratory glassware and utensils.

To perform the experimental work described in this study, the following was needed:

- BactoCount IBC™ 3.0;
- Instruction and method implementation;
- Statistical expertise.

2.2. Performance characteristics of the alternative method

All **performance characteristics** of the BactoCount IBC™ 3.0 for TBC were evaluated during the MicroVal validation study with raw cow milk. **All conditions of the tests and detailed results are available in the Validation report** (MicroVal study number 2021LR97).

- The linearity of the instrument for TBC was evaluated in raw cow milk containing up to 5 000x10^3 CFU/mL. The instrument was considered as linear up to this value.
- The instrument was stable in the working day for TBC in raw cow milk containing 4.7 Log_{10} CFU/mL to 5.5 Log_{10} CFU/mL.
- The carry-over effect of the instrument for TBC was lower than the acceptability limits defined in the ISO 16297 at all levels tested (46x10^3; 143x10^3; 284x10^3 and 1 600x10^3 CFU/mL).
- The lower limit of quantification for TBC was defined at **5160 CFU/mL** in raw cow milk.

2.3. Repeatability and accuracy of BactoCount IBC™ 3.0 for TBC

The evaluation was performed in **goat herd milk samples**. Accuracy trials were carried out against ISO 4833-1:2013+Amd 1:2022.

2.3.1. Repeatability (according to ISO 16297 § 5.6.2)

Repeatability should be estimated with a large number of measurements in duplicate performed on samples covering the entire measuring range.

2.3.1.1. Measurement protocol and calculations

The standard deviation of repeatability (s_r) of the BactoCount IBC™ 3.0 was calculated from testing results with **342 goat herd milk samples** representative for different total bacterial count levels as shown in Table 1. *Note that 2 outlier samples were eliminated by COCHRAN 5% according to the ISO 16297 and ISO 5725-2 (9).* These problems of repeatability on these samples may be due to a sample exchange during measurement or an uncommon problem of sampling. Raw data are presented in Annex 1.

Table 1: Raw goat milk samples selected for estimation of the repeatability of the BactoCount IBC™ 3.0 for total bacterial count; Values in brackets represent the number of samples without sample elimination. Sorting of samples on the concentration obtained with the IBC™ 3.0.

Bacterial count level (Log_{10} CFU/mL)	Number of goat herd milk samples
3.7 – 4.7	**56** (57)
4.7 – 5.7	**194** (195)
5.7 – 6.7	**92** (92)
Total number of samples	**342** (344)

All raw goat's milk samples were measured in duplicate (n=2) with BactoCount IBC™ 3.0. The standard deviation of repeatability (s_r) was calculated for each cell count level as:

$$s_r = \left(\sum w_i^2 / 2q\right)^{1/2}$$

With $i(w_i = |x_{1i} - x_{2i}|)$

The calculations were performed without any transformation.

2.3.1.2. Results

The **standard deviation of repeatability (s_r)** of BactoCount IBC™ 3.0 for enumeration of total bacteria was calculated for goat herd milk. The results and the acceptability values are given in Table 2. Only 6 samples are in the lower range of concentration. Results obtained in this range are within specifications but not used to conclude.

Table 2 : The standard deviation of repeatability (sr) of the BactoCount IBC™ 3.0 for enumeration of total bacteria calculated per bacterial count level and acceptability values according to ISO 16297 and EU-RL MMP document; Values in brackets represent the results without sample elimination.

Bacterial count level (Log_{10} CFU/mL)	Number of samples	s_r goat herd milk samples: Mean level samples (Log_{10} CFU/mL)	s_r goat herd milk samples: s_r	s_r acceptability values according to ISO 16297 and EU-RL MMP document
< 4.3	**6** (7)	**4.2** (4.3)	**0.08** (0.15)	**0.12**
≥ 4.3	**336** (337)	**5.3** (5.3)	**0.05** (0.05)	**0.09**

2.3.1.3. Conclusion

The repeatability of the BactoCount IBC™ 3.0 for total bacterial count in raw goat milk complies with the requirement of ISO 16297 and the EU-RL MMP document in the higher range of concentration (≥ 4.3 Log_{10} CFU/mL).

2.3.2. Accuracy (according to ISO 16297 § 6.4.4)

The accuracy of the alternative method is based on the **residual standard deviation, s_{yx},** of the simple linear regression of the instrumental results obtained in duplicate, y, and the results obtained with the anchoring method (ISO 4833 in this study) in duplicate, x.

2.3.2.1. Measurement protocol and calculations

The residual standard deviation of the BactoCount IBC™ 3.0 for enumeration of total bacteria was evaluated at **different total bacterial count levels through comparison with the anchoring method**.

It was calculated with 337 unpreserved raw goat herd milk samples as shown in Table 3. *Note that 5 outliers samples were eliminated (difference between the two methods higher than 2.58 times the residual standard deviation according to ISO 16297 and ISO 21187:2021). These differences concerning these outlier samples may be due to the presence of a particular flora that can gave different results of numeration between methods based on difference principles.* Raw data are presented in Annex 1.

All samples were measured in duplicate with the evaluated instrument.

Table 3: Raw goat's milk samples selected for determination of residual standard deviation of the BactoCount IBC™ 3.0 for enumeration of total bacteria; Values in brackets represent the number of samples without elimination. Sorting of samples on the concentration obtained with the ISO 4833.

Bacterial count level (Log_{10} CFU/mL)	Number of goat herd milk samples
3.7 – 4.7	**147** (149)
4.7 – 5.7	**126** (126)
5.7 – 6.7	**64** (67)
Total number of samples	**337** (342)

The relationship between results with the evaluated instrument was visually inspected by plotting the results obtained with the BactoCount IBC™ 3.0 on the y-axis and the results obtained with the ISO 4833 method on the x-axis.

A linear regression was applied and the standard deviation of individual results s_{yx} was determined.

For each sample, the logarithmic difference between the methods was calculated as:

$$\Delta C_{2i} = C_{alt,i} - C_{anch,i}$$

where:

ΔC_{2i} = difference between results obtained with the 2 methods for the ith sample
$C_{alt,i}$ = the result of the alternative method for the ith sample
$C_{anch,i}$ = the result of the anchoring method for the ith sample

For each total bacterial count level (interval of 0.5 $\log_{10}$ CFU/mL), following calculate were performed:

- The mean and standard deviation of results of the anchoring method
- The mean of the logarithmic difference, $\overline{\Delta C_{2\imath}}$
- The standard deviation of logarithmic difference, $s_{\Delta C_{2i}}$
- The limit of logarithmic confidence (95%) as $\overline{\Delta C_{2\imath}} \pm 1.96 s_{\Delta C_{2i}}$

Results were plotted on a graph as an accuracy profile.

2.3.2.2. Results

The accuracy of BactoCount IBC 3.0 was evaluated against ISO 4833 with a linear regression.

The **standard deviation of individual results s_{yx} was 0.31 $\log_{10}$ CFU/mL** (0.32 without sample elimination) and **complies with the limit of 0.40 $\log_{10}$ CFU/mL** defined in the ISO 16297 and the EU-RL MMP document.

The correlation between the evaluated models is visualized in Figure 1. Moreover, the accuracy profile was determined and presented in Figure 2. The positive ΔC_2 for all samples and the negative slope of the accuracy profile may reflect a shift in results (function of concentration) which can be linked to the universal conversion equation used to convert IBC into CFU/ml in this study.

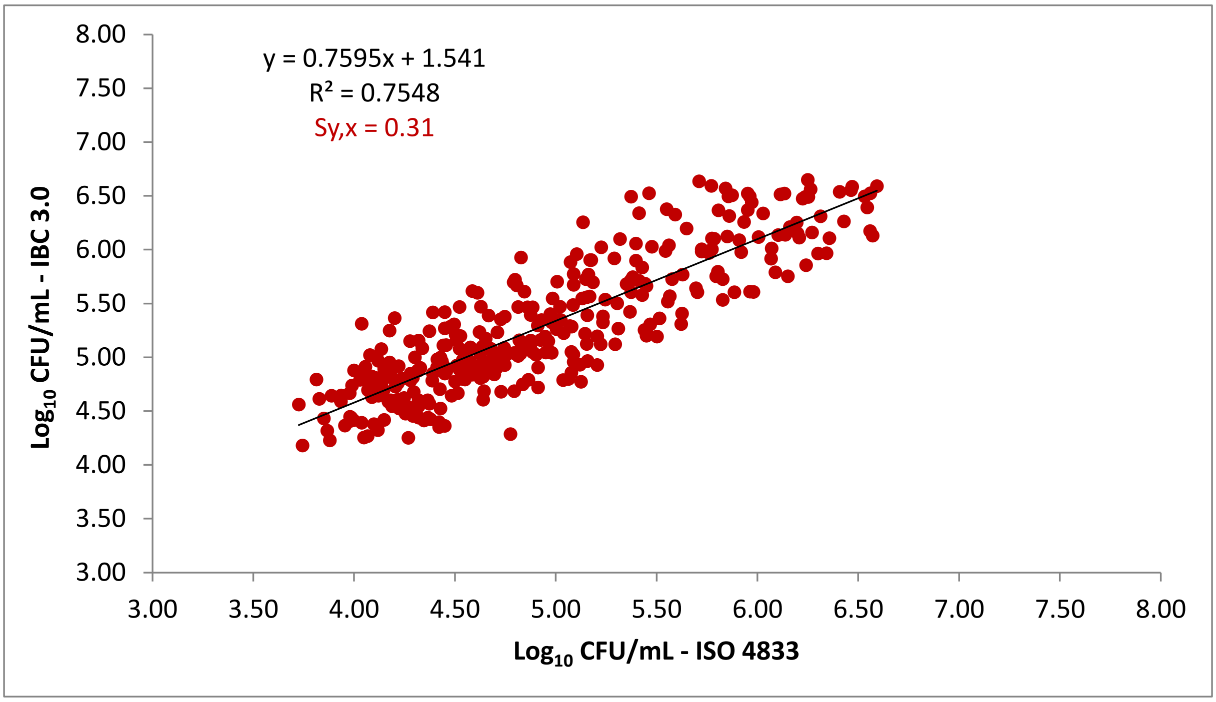


Figure 1: Relationship between BactoCount IBC^{TM} 3.0 and ISO 4833 for raw goat milk samples.

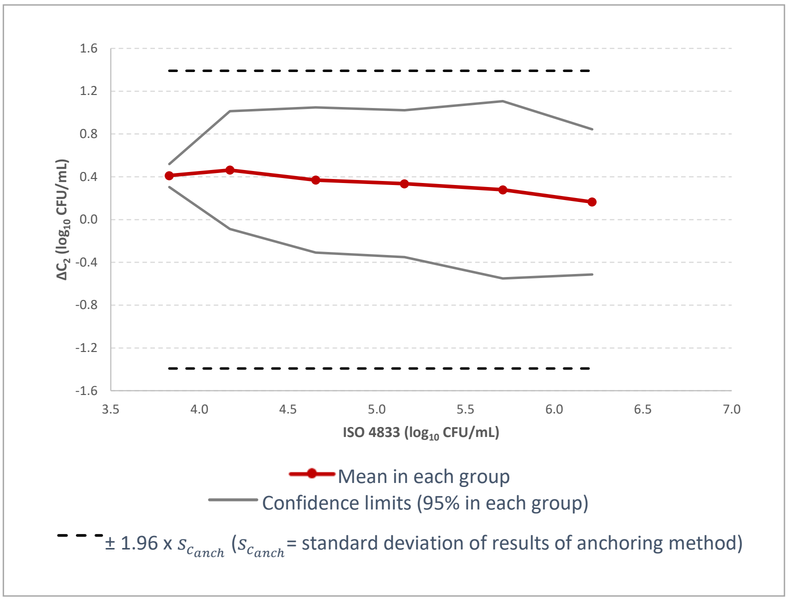


Figure 2: Accuracy profile of BactoCount IBC^{TM} 3.0 for enumeration of total bacteria in raw goat milk.

2.3.2.3. Conclusion

The Accuracy of the IBC^{TM} 3.0 for the enumeration of total bacteria in raw goat milk complies with the requirement of ISO 16297 and the EU-RL MMP document.

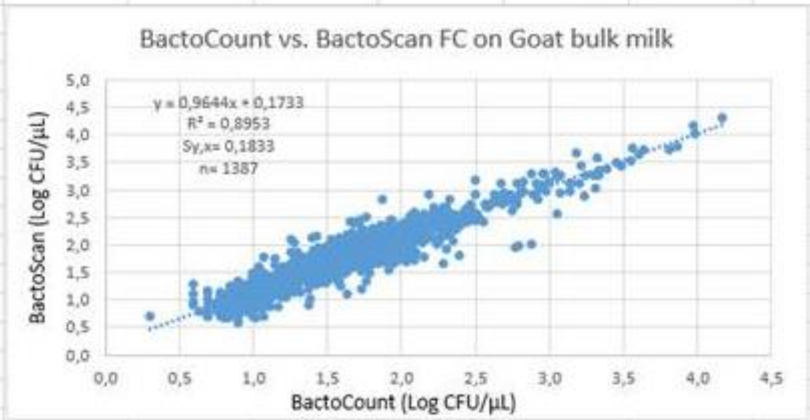
2.4. Interference of SCC on TBC prediction

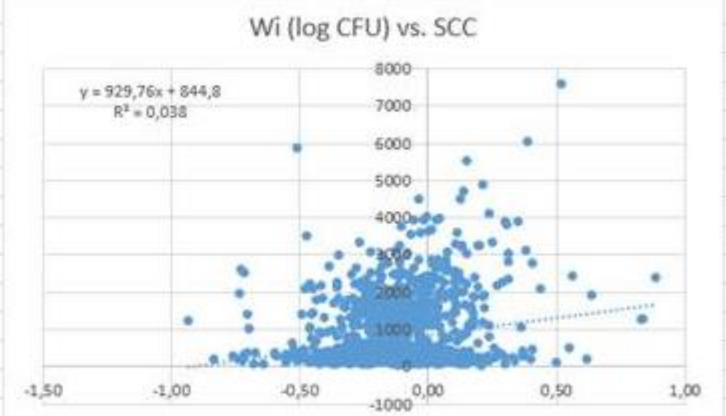
Two experiments have been conducted successfully to evaluate the interference of SCC on TBC prediction using Bactocount instruments (automatic and manual version).

- The first one by analyzing SCC commercial reference materials (until around 2 x 10^6 somatic cells / ml). the results are presented in the tab below and show the non interference of SCC level (in the range tested) on the TBC prediction.

Lot n°	SCC REF	LogCFU
QLIP 2340291 EXP:03/01/24 C1	179	5,35
	179	5,33
QLIP 2340291 EXP:03/01/24 C2	324	5,35
	324	5,37
QLIP 2340291 EXP:03/01/24 C3	469	5,35
	469	5,36
QLIP 2340291 EXP:03/01/24 C4	614	5,34
	614	5,35
QLIP 2340291 EXP:03/01/24 C5	759	5,32
	759	5,33
QLIP 2340291 EXP:03/01/24 C6	1485	5,32
	1485	5,32
QLIP 2340291 EXP:03/01/24 C7	1920	5,31
	1920	5,28

- The second experiment have been conducted from a statistical approach from the data set obtained in the step 2 of the final validation requested by the CNIEL in France. The Bactocount IBC 3.0 results have been compared to the Bactoscan FC+ on 1387 goat milks. The differences within the 2 instruments has been plotted vs the SCC of the samples.
  At first, we can observe the residual standard deviation of the relationship between the results of both instruments is 0.1833 Log.
  Then the correlation between the differences (within the 2 instruments) and SCC is very low (0.038) and the differences within the 2 instruments are balanced on both sides of the line for the same SCC levels. These observations allow to confirm the non-interference of SCC on TBC prediction by the Bentley Bactocount IBC3.0 in the SCC range tested.





The two experiments conducted on Bentley Bactocount instruments confirm the non interference of SCC level on TBC prediction, at least until the upper level of the range tested around $5x10^6$ SCC / ml.

2.5. Conclusion of the method comparison study for TBC

BactoCount IBC^{TM} 3.0 **performance characteristics** for enumeration of total bacteria according to ISO 16297 have not been evaluated again in raw goat milk. All data are available in the MicroVal Validation report corresponding to the study n° 2021LR97.

Concerning the **overall accuracy evaluation** of BactoCount IBC^{TM} 3.0 for enumeration of total bacteria according to ISO 16297 in goat herd milk samples:

- **Repeatability** for samples ≥ 4.3 $\log_{10}$ CFU/mL:

  $$s_r = 0.05$$

- **Accuracy** for all goat herd milk samples:

  $$s_{yx} = 0.31 \log_{10} \text{CFU/mL}$$

On figure 3, all data (couple reference / IBC) obtained during MicroVal validation studies conducted at ACTALIA Cecalait in 2022-2023 on cow, sheep and goat milks samples have been plotted (in orange) on the Bentley's data set of the universal equation (blue). The two populations of data (orange and blue) are mixed, meaning the universal conversion equation is appropriate for milk from the 3 species. *However, user laboratories should verify the applicability of the universal conversion equation with routine analyzed samples. If not applicable, they have to develop a separate conversion equation according to ISO 21187. It is also necessary to regularly evaluate the validity of an applied conversion equation and to maintain compliance with applicable regulations.*

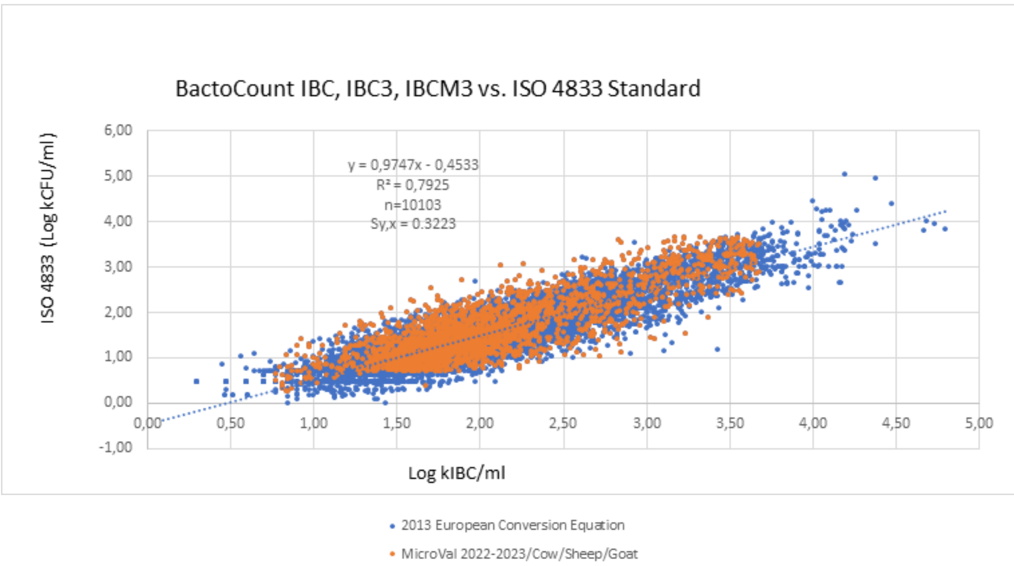


Figure 3: Graphical representation of results obtained for the definition of the Conversion equation (●) and during the various MicroVal validation studies conducted on cow, sheep and goat milks in 2022/2023 (●)

Extension of METHOD COMPARISON STUDY

FOR ENUMERATION OF

SOMATIC CELLS (SCC)

In raw goat milk

3. Extension of Method comparison study for enumeration of Somatic Cell (SCC)

3.1. Materials and equipment used

- Goat herd milk samples;
- Stock and working solutions for BactoCount IBC™ 3.0 and Somacount™ FCM, prepared according to manufacturer's instructions;
- RBS 2% solution (PN 9008);
- SCC incubation / dye solution;
- Somatic cells SRM (Standard Reference Material from Actalia Cecalait);
- Refrigerator at 0-4°C;
- Water bath at 40±2°C;
- Standard laboratory glassware and utensils.

To perform the experimental work described in this study, the following was needed:

- BactoCount IBC™ 3.0;
- Somacount™ FCM (ICAR certified according ISO 8196-3; certificate n°2020/7);
- Instruction and method implementation;
- Statistical expertise.

3.2. Performance characteristics of the alternative method

All **performance characteristics** of the BactoCount IBC™ 3.0 for SCC were evaluated during the MicroVal validation study in raw cow milk. **All conditions of the tests and detailed results are available in the Validation report** (MicroVal study number 2021LR97).

- The linearity of the instrument for SCC was evaluated with raw cow milk containing up to 2 500x10^3 cells/mL. The instrument was considered as linear up to this value.
- The instrument was stable in the working day for SCC in raw cow milk containing 73x10^3 to 1 439x10^3 cells/mL.
- The carry-over effect of the instrument for SCC was lower than the acceptability limits defined in the ISO 8196-3 (2022) at all levels tested (501x10^3; 988x10^3; 1 477x10^3 cells/mL).
- The lower limit of quantification for SCC was defined at **10.2x10^3 cells/mL** in raw cow milk.

3.3. Repeatability and accuracy of BactoCount IBC™ 3.0 for SCC

The **overall accuracy** is the sum of the **repeatability error**, the **accuracy error** and the **calibration error**.

With raw milk, each part of the overall precision is measured by analysis of herd milk samples of the specified animal species. The evaluation should be performed under conditions equivalent to the intended routine use.

The same samples have been analyzed in duplicates for repeatability evaluation and for accuracy evaluation (average of the two replicates).

3.3.1. Calibration (according to ISO 8196-3 § 5.2.2.2.5.3)

Calibration of BactoCount IBC^{TM} 3.0 and SomaCount^{TM} FCM were performed according to the manufacturer's recommendations with somatic cells Standard Reference Material from Actalia Cecalait (traceable to IRMM CRM). The Standard Reference Materials were used to calibrate and check the calibration.

Results of measurement of CECALAIT Standard Reference Materials of 10 milk samples with somatic cells concentration from 0 to 1 800 x10^3 cells/mL and the linear regressions of the results obtained with the SomaCount^{TM} FCM and the IBC^{TM} 3.0 are represented in Figure 4 and Figure 5 respectively.

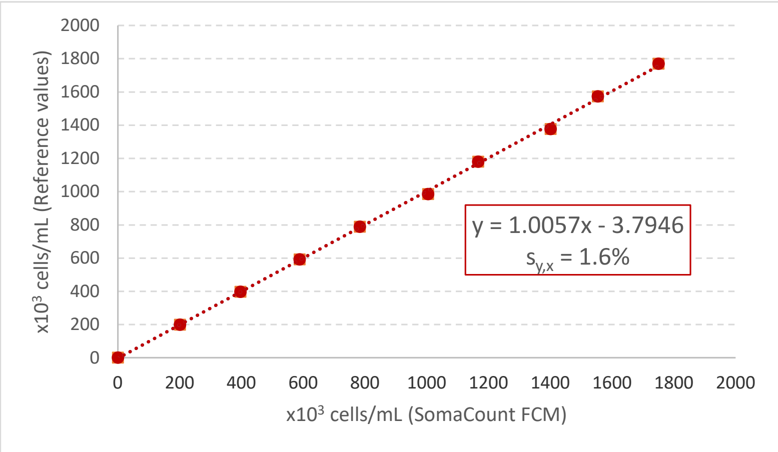


Figure 4: Linear regression of SomaCount^{TM} FCM measurements of SRM

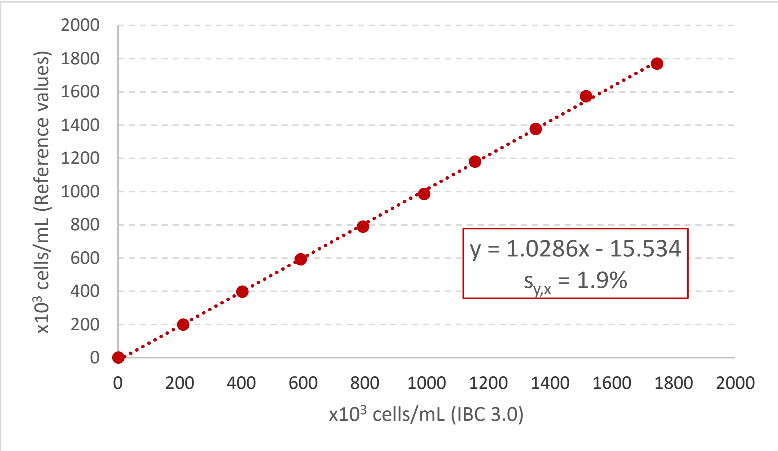


Figure 5: Linear regression of BactoCount IBC^{TM} 3.0 measurements of SRM

The slope of the linear regression and the relative bias calculated for the two instruments were presented in the Table 4. **All parameters were in accordance with the ISO 8196 requirements for the 2 instruments**.

Table 4: Slope of the linear regression (b), relative bias ($\bar{d}_{rel}$) and residual standard deviation ($s_{y,x}$) calculated for the two instruments

Parameters	Somacount™ FCM	IBC 3.0	Acceptability values according to ISO 8196-3
Slope of the linear regression (*b*)	**1.006**	**1.030**	**1 ±0.05**
relative bias ($\bar{d}_{rel}$)	**- 0.14%**	**-1.07%**	**± 5%**
Residual standard deviation ($s_{y,x}$)	**1.6%**	**1.9%**	**-**

3.3.2. Repeatability (according to ISO 8196-3 § 5.2.2.2.4)

Repeatability is the primary criterion for determining whether a method produces **stable results in accordance with the user's requirements**. It is the major element of internal quality control. Therefore, each new instrument must meet a maximum limit of repeatability value specified in the applicable International Standard to meet the accreditation criteria.

3.3.2.1. Measurement protocol and calculations

The standard deviation of repeatability (s_r) of the BactoCount IBC™ 3.0 was calculated from testing results with 169 raw goat herd milk samples representative for different somatic cell count levels as shown in Table 5. *Note that 3 outlier samples were eliminated by COCHRAN 5% according to ISO 5725-2 (9).* These problems of repeatability on these samples may be due to a sample exchange during measurement or an uncommon problem of sampling. Only 5 samples are in the lower range of concentration (450-750x10^3 cells/mL). Results obtained in this range are therefore not used to conclude.

Table 5: Raw goat milk samples selected for estimation of the repeatability of the Bactocount IBC™ 3.0 for enumeration of somatic cells; Values in brackets represent the number of samples without elimination. AL = acceptability limits calculated on the basis of the measured cell count levels and the values in Table 2 in ISO 13366-2 currently under review

Cell count level	Number of raw milk samples	Mean level samples	S_r			*r*	
x10^3 cells/mL	-	x10^3 cells/mL	x10^3 cells/mL	%	AL (%)	x10^3 cells/mL	AL (x10^3 cells/mL)
450 – 750	**5** (5)	**665** (665)	**13.3** (13.3)	**2.0%** (2.0%)	**3.0%**	**38** (38)	**55.9**
750 – 1 500	**102** (105)	**1 174** (1 175)	**20.3** (25.0)	**1.7%** (2.1%)	**2.4%**	**57** (71)	**80.0**
1 500 – 2 500	**62** (62)	**1 779** (1 779)	**26.6** (26.6)	**1.5%** (1.5%)	**2.0%**	**75** (75)	**84.0**
Total number of samples	**169** (172)	**1 381** (1 378)	**22.7** (25.4)	**1.6%** (1.8%)	-	**64** (72)	-

3.3.2.2. Conclusion

The standard deviation of repeatability (s_r) for the enumeration of somatic cells in raw goat milk is lower than the limit defined in the ISO 13336-2 for samples containing 750 to 2500x10^3 cells/mL.

3.3.3. Accuracy (according to ISO 8196-3 § 5.2.2.2.5.2)

The **accuracy** of the alternative method is based on the **residual standard deviation, s_{yx},** of the simple linear regression of the instrumental results obtained in duplicate, x, and the reference results obtained in duplicate, y.

3.3.3.1. Measurement protocol and calculations

The residual standard deviation of the Bactocount IBC^{TM} 3.0 for enumeration of somatic cells was evaluated at different somatic cell count levels **through comparison with the Somacount^{TM} FCM**. It was calculated with 163 raw goat milk samples as shown in Table 6. *Note that 6 outlier samples were eliminated (difference between the two methods higher than 2.58 times the residual standard deviation according to ISO 21187:2021).* Only 4 samples are in the lower range of concentration (450-750x10^3 cells/mL). Results obtained in this range are therefore not used to conclude.

All samples were measured in duplicate with the two instruments.

Table 6: Raw goat milk samples used for determination of residual standard deviation of the Bactocount IBC^{TM} 3.0 for enumeration of somatic cells; Values in brackets represent the number of samples without elimination.

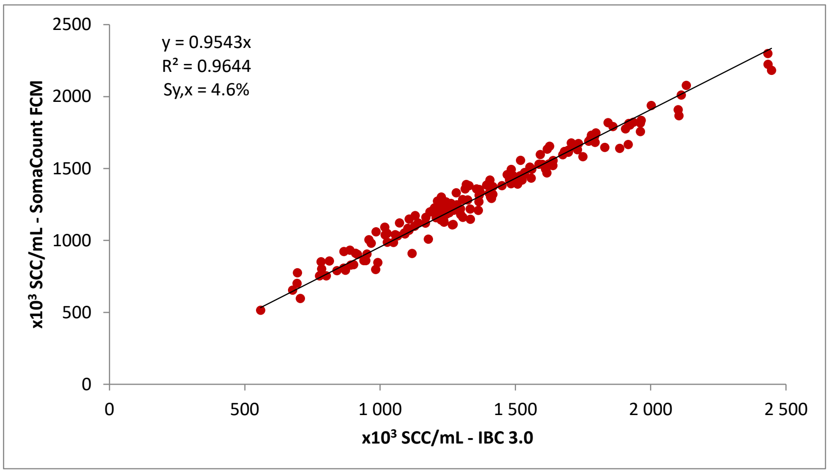
Cell count level	Number of raw milk samples	Mean level samples	$S_{y,x}$		
x10^3 cells/mL	-	x10^3 cells/mL	x10^3 cells/mL	%	AL (%)
450 – 750	**4** (6)	**616** (614)	**60.3** (70.6)	**9.8%** (11.5%)	**8%**
750 – 1 500	**111** (113)	**1 167** (1 171)	**61.5** (69.2)	**5.3%** (5.9%)	**8%**
1 500 – 2 500	**48** (50)	**1 730** (1 724)	**55.2** (69.9)	**3.2%** (4.1%)	**8%**
Total number of samples	**163** (169)	**1 319** (1 315)	**61.0** (83.2)	**4.6%** (6.3%)	**8%**

The accuracy calculated is lower than the limits defined in the ISO 8196-3 for samples containing 750 to 2500x10^3 cells/mL. The $S_{y,x}$ calculated for the entire sample set is lower than the acceptability limit.

The accuracy of Bactocount IBC^{TM} 3.0 for SCC in goat milk was evaluated against Somacount^{TM} FCM with a linear regression.

The correlation between the evaluated models is visualized in Figure 6.

Figure 6: Relationship between Bactocount IBC^{TM} 3.0 and Somacount^{TM} FCM for SCC in raw goat milk samples (interception defined = 0)



3.3.3.2. Conclusion

Accuracy of the IBC^{TM} 3.0 for the enumeration of somatic cells in raw goat milk complies with the requirement of ISO 13366-2 and ISO 8196-3.

4. Final conclusion of the validation study

All performance characteristics of the BactoCount IBC^{TM} 3.0 for TBC and SCC were evaluated during the MicroVal validation study on raw cow milk and complies with ISO 16297 (2020), ISO 8196-3 (2022) and EURL MMP documents (MicroVal study number 2021LR97).

In the present study, the comparison with the reference method (ISO 4833) for TBC and with the already validated SomaCount^{TM} FCM for SCC revealed that the BactoCount IBC^{TM} 3.0 can be used for enumeration of total bacteria and somatic cells in raw goat milk.

5. References

1. ISO 16297|IDF 161:2020 Milk – Bacterial count – Protocol for the evaluation of alternative methods.

2. ISO 21187:2021 Milk – Quantitative determination of bacteriological quality – Guidance for establishing and verifying a conversion relationship between routine method results and anchor method results.

3. ISO 16140-2:2016 - Microbiology of the food chain — Method validation — Part 2: Protocol for the validation of alternative (proprietary) methods against a reference method

4. ISO 8196-3|IDF 128-3:2022 Milk – Definition and evaluation of the overall accuracy of alternative methods of milk analysis – Part 3: Protocol for the evaluation and validation of alternative quantitative methods of milk analysis.

5. EN ISO 13366-2|IDF 148-1:2006 Milk – Enumeration of somatic cells – Part 2: Guidance on the operation of fluoro-optoelectronic counters.

6. ISO 4833-1:2013+Amd 1:2022 Microbiology of the food chain — Horizontal method for the enumeration of microorganisms — Part 1: Colony count at 30 °C by the pour plate technique — Amendment 1: Clarification of scope

7. Comparison of the analytical instruments BactoCount and BactoScan FC using raw milk samples. Final report September 2006; Bühlmann, G.; 2006 ; https://docplayer.org/69900419-Comparison-of-the-analytical-instruments-bactocount-and-bacto-scan-fc-using-raw-milk-samples-final-report-september-2006.html

8. EU-RL MMP document – Validation criteria of instrumental methods for enumeration of total flora in raw milk, version 2, 21 December 2011.

9. ISO 5725-2:2019 - Accuracy (trueness and precision) of measurement methods and results — Part 2: Basic method for the determination of repeatability and reproducibility of a standard measurement method

ANNEX 1: RAW DATA OF TBC IN LOG_{10} CFU/ML FOR BACTOCOUNT IBC™ 3.0 AND THE REFERENCE METHOD (ISO 4833)

Samples highlighted in blue = eliminated by COCHRAN 5%
Samples highlighted in orange = differences between methods > 2.58 the standard deviation

	IBC3.0 (Log_{10} CFU/mL)			ISO 4833 (Log_{10} CFU/mL)
Samples	R1	R2	Mean	
230112-1	5.75	5.74	5.75	5.38
230112-2	5.51	5.56	5.53	5.83
230112-3	5.46	5.48	5.47	4.63
230112-5	6.56	6.58	6.57	5.84
230112-6	5.32	5.38	5.35	4.73
230112-7	5.59	5.62	5.61	5.37
230112-8	5.49	5.45	5.47	4.81
230112-9	5.04	5.19	5.11	4.46
230112-10	5.22	5.35	5.28	5.08
230112-11	5.45	5.48	5.47	4.89
230112-12	6.48	6.51	6.50	5.86
230112-13	4.28	4.56	4.42	4.15
230112-15	6.10	6.10	6.10	5.77
230112-16	5.47	5.47	5.47	4.52
230112-17	5.03	4.97	5.00	4.30
230112-18	5.36	5.34	5.35	5.00
230112-20	5.42	5.34	5.38	4.75
230112-21	5.32	5.38	5.35	5.01
230112-22	5.71	5.64	5.67	5.09
230112-23	5.47	5.47	5.47	5.02
230112-25	6.59	6.60	6.59	5.77
230112-27	6.07	6.11	6.09	5.91
230112-28	5.69	5.73	5.71	5.41
230112-29	5.94	5.90	5.92	5.29
230112-31	5.20	5.10	5.15	4.96
230112-32	6.54	6.51	6.52	5.46
230112-33	6.57	6.54	6.55	6.46
230112-34	5.06	5.01	5.04	4.79
230112-35	6.51	6.47	6.49	6.25
230112-36	6.03	6.02	6.03	5.48
230112-38	5.00	4.90	4.95	4.64
230112-40	5.21	5.11	5.16	4.51
230112-42	6.39	6.36	6.38	5.55
230112-45	5.73	5.73	5.73	5.58
230112-46	5.25	5.29	5.27	4.45
230112-47	5.63	5.68	5.66	4.30
230112-48	4.91	4.94	4.92	4.59
230112-49	4.90	4.86	4.88	4.56

230112-50	6.37	6.36	6.37	5.95
230113-51	6.52	6.53	6.52	5.95
230113-52	5.69	5.84	5.77	5.16
230113-54	5.52	5.60	5.56	5.15
230113-55	5.81	5.86	5.83	5.43
230113-58	6.00	6.00	6.00	5.77
230113-59	6.10	6.14	6.12	5.85
230113-60	6.32	6.35	6.34	6.03
230113-62	6.47	6.48	6.47	6.22
230113-64	5.72	5.73	5.73	5.15
230113-65	6.04	6.04	6.04	5.56
230113-66	6.31	6.31	6.31	5.86
230113-67	4.58	4.62	4.60	4.18
230113-68	6.50	6.53	6.51	6.11
230113-69	5.76	5.79	5.77	5.09
230113-70	5.16	5.15	5.16	4.82
230113-72	5.47	5.46	5.46	4.86
230113-73	5.05	5.10	5.08	4.83
230113-74	5.39	5.40	5.39	4.88
230113-75	6.48	6.51	6.50	5.96
230113-76	5.71	5.68	5.70	4.79
230113-77	6.63	6.64	6.64	5.71
230113-78	4.83	4.80	4.81	4.29
230113-80	5.32	5.37	5.35	5.02
230113-81	5.05	5.11	5.08	4.52
230113-82	5.56	5.53	5.55	4.98
230113-83	5.35	5.34	5.35	4.93
230113-84	6.34	6.34	6.34	5.41
230113-85	6.07	6.05	6.06	5.40
230113-86	5.68	5.68	5.68	5.35
230113-87	4.89	4.96	4.92	4.51
230113-88	4.82	4.86	4.84	4.60
230113-89	4.87	4.89	4.88	4.31
230113-90	5.69	5.71	5.70	5.19
230113-91	5.59	5.54	5.56	5.17
230113-92	6.37	6.36	6.37	5.81
230113-93	5.67	5.27	5.47	4.75
230113-95	5.28	5.52	5.40	4.97
230113-96	4.93	4.87	4.90	4.71
230113-97	5.03	5.00	5.02	4.75
230113-98	6.43	6.45	6.44	5.97
230113-99	5.09	5.10	5.10	4.64
230113-100	5.26	5.21	5.23	4.62
230120-2	5.18	5.17	5.17	4.65
230120-3	4.84	4.92	4.88	4.00

230120-5	4.28	4.18	4.23	3.88
230120-7	5.21	5.28	5.24	4.37
230120-8	6.34	6.28	6.31	6.31
230120-10	5.51	5.51	5.51	4.06
230120-11	5.41	5.40	5.41	5.63
230120-12	4.91	4.91	4.91	4.06
230120-13	5.21	5.25	5.23	4.71
230120-14	4.64	4.64	4.64	4.48
230120-15	4.54	4.59	4.57	4.37
230120-16	6.65	6.64	6.65	6.25
230120-17	5.43	5.35	5.39	4.67
230120-19	4.70	4.69	4.69	4.15
230120-20	5.62	5.67	5.64	5.70
230120-21	4.97	4.93	4.95	4.18
230120-23	5.23	5.35	5.29	5.07
230120-25	6.56	6.57	6.56	6.26
230120-29	5.33	5.29	5.31	5.00
230120-30	5.37	5.25	5.31	4.04
230120-31	4.56	4.65	4.60	4.22
230120-32	5.84	5.92	5.88	5.07
230120-33	5.52	5.61	5.57	5.57
230120-34	6.57	6.62	6.59	6.59
230120-36	4.86	4.84	4.85	4.28
230120-41	5.23	5.22	5.23	5.04
230120-42	5.60	5.63	5.62	4.59
230120-44	4.94	4.83	4.89	4.52
230120-45	4.96	4.64	4.80	4.24
230120-48	5.59	5.62	5.61	5.70
230120-50	5.09	5.13	5.11	4.44
230120-52	4.81	4.83	4.82	4.64
230120-53	6.01	6.00	6.00	5.72
230120-54	4.90	4.91	4.90	4.91
230120-55	4.38	4.26	4.32	3.87
230120-56	4.08	4.28	4.18	3.74
230201-1	4.53	4.36	4.45	3.98
230201-5	4.86	4.71	4.78	4.39
230201-6	5.08	5.07	5.08	4.13
230201-7	5.59	5.63	5.61	4.85
230201-8	4.04	4.52	4.28	3.77
230201-9	4.90	4.83	4.87	4.46
230201-10	5.71	5.70	5.70	5.01
230201-11	5.69	5.68	5.68	5.44
230201-12	4.58	4.62	4.60	4.32
230201-13	4.32	4.32	4.32	4.12
230201-14	4.78	4.70	4.74	3.99

230201-15	5.21	5.21	5.21	4.50
230201-16	4.26	4.28	4.27	4.07
230201-17	4.85	4.93	4.89	4.15
230201-18	5.89	5.90	5.90	5.40
230201-19	4.46	4.41	4.44	4.32
230201-20	5.70	5.63	5.66	5.45
230201-21	4.41	4.46	4.44	3.99
230201-22	5.29	5.30	5.30	4.91
230201-23	4.46	4.45	4.45	4.29
230201-24	5.70	5.75	5.73	5.83
230201-25	4.49	4.57	4.53	4.28
230201-26	5.12	5.18	5.15	4.82
230201-27	5.55	5.60	5.58	5.43
230201-28	4.38	4.34	4.36	4.45
230201-29	4.66	4.62	4.64	4.12
230201-30	4.99	5.00	5.00	4.43
230201-31	4.36	4.46	4.41	4.35
230201-32	4.81	4.81	4.81	4.10
230201-33	4.71	4.70	4.70	4.13
230201-34	4.66	4.70	4.68	4.73
230201-35	4.43	4.36	4.40	4.42
230201-36	4.54	4.54	4.54	4.32
230201-37	4.96	4.98	4.97	4.54
230201-38	5.51	5.49	5.50	5.30
230201-39	5.15	5.09	5.12	5.29
230201-40	5.12	5.06	5.09	4.58
230202-41	4.51	4.54	4.52	4.43
230202-42	4.80	4.78	4.79	4.10
230202-43	5.19	5.20	5.19	5.21
230202-45	6.25	6.26	6.26	5.93
230202-46	5.63	5.71	5.67	4.81
230202-47	5.12	5.19	5.16	4.88
230202-48	4.85	4.85	4.85	4.45
230202-49	5.06	5.11	5.08	4.34
230202-50	5.40	5.43	5.42	4.39
230202-52	5.17	5.44	5.31	4.50
230202-53	4.96	4.96	4.96	4.44
230202-54	4.94	4.97	4.96	4.54
230202-55	5.17	5.21	5.19	4.95
230202-56	4.58	4.63	4.61	4.64
230202-57	5.73	5.77	5.75	5.80
230202-58	6.12	6.15	6.14	6.14
230202-59	4.53	4.64	4.59	4.17
230202-60	5.36	5.37	5.36	4.20
230202-61	5.59	5.61	5.60	4.61

230202-62	4.23	4.34	4.29	4.77
230202-64	6.10	6.10	6.10	5.32
230202-65	5.00	5.09	5.05	5.08
230202-66	5.96	5.89	5.93	4.83
230202-67	4.73	4.95	4.84	4.70
230202-68	5.13	5.19	5.16	4.92
230202-69	4.20	4.30	4.25	4.27
230202-70	4.65	4.72	4.68	4.64
230202-71	4.89	4.87	4.88	4.18
230202-72	5.24	5.16	5.20	4.53
230202-73	4.88	4.88	4.88	4.32
230202-74	5.28	5.25	5.27	5.31
230202-76	4.91	5.16	5.03	4.82
230202-77	6.09	6.12	6.10	5.79
230202-78	4.45	4.26	4.35	4.42
230202-79	5.34	5.41	5.38	5.23
230202-80	5.27	5.34	5.31	5.62
230202-81	4.81	4.74	4.77	5.13
230202-82	4.79	4.79	4.79	4.28
230202-83	5.36	5.42	5.39	5.16
230202-84	6.22	6.20	6.21	6.16
230202-85	4.89	4.81	4.85	4.39
230202-86	5.00	5.09	5.04	4.56
230202-87	5.23	5.21	5.22	5.14
230202-88	5.05	4.92	4.98	4.41
230202-89	5.31	5.34	5.32	5.23
230202-90	4.45	4.43	4.44	4.37
230202-91	4.43	4.40	4.41	4.00
230202-92	5.78	5.80	5.79	6.09
230202-93	4.40	4.41	4.41	3.98
230323-17	6.25	6.27	6.26	5.13
230323-23	6.50	6.49	6.49	5.37
230323-24	6.22	6.18	6.20	5.65
230323-30	6.26	6.39	6.33	5.59
230323-31	5.91	6.13	6.02	5.23
230414-1	5.05	5.05	5.05	4.89
230414-2	4.63	4.62	4.63	3.92
230414-3	5.54	5.55	5.55	5.13
230414-4	4.95	4.87	4.91	4.64
230414-5	4.70	4.71	4.70	4.43
230414-6	4.87	4.81	4.84	4.59
230414-7	4.62	4.56	4.59	3.93
230414-8	5.96	5.96	5.96	5.10
230414-9	5.20	5.26	5.23	5.45
230414-10	5.61	5.59	5.60	5.89

230414-11	5.12	5.12	5.12	4.88
230414-12	4.65	4.68	4.67	3.98
230414-13	4.81	4.80	4.81	4.63
230414-14	4.59	4.53	4.56	3.73
230414-15	4.76	4.83	4.79	3.81
230414-16	5.24	5.27	5.25	5.44
230414-17	4.43	4.41	4.42	4.38
230414-18	5.24	5.28	5.26	5.00
230414-19	4.94	4.89	4.92	4.65
230414-20	4.79	4.79	4.79	4.55
230414-21	4.94	4.87	4.91	4.41
230414-22	4.81	4.77	4.79	4.03
230414-23	4.48	4.28	4.38	4.10
230414-24	4.26	4.26	4.26	4.05
230414-26	4.69	4.68	4.69	4.79
230414-28	4.53	4.56	4.54	4.19
230414-29	4.65	4.59	4.62	4.25
230414-30	5.76	5.75	5.75	6.15
230414-31	4.30	4.43	4.37	3.95
230414-32	4.70	4.65	4.68	4.30
230414-33	5.11	5.05	5.08	4.68
230414-34	5.04	5.05	5.05	4.95
230421-1	5.22	5.18	5.20	5.45
230421-2	5.39	5.45	5.42	4.45
230421-3	4.60	4.62	4.61	3.83
230421-4	4.72	4.72	4.72	4.91
230421-5	5.02	5.02	5.02	4.08
230421-6	4.90	4.94	4.92	4.15
230421-7	5.15	5.10	5.12	5.15
230421-9	4.81	4.86	4.84	4.05
230421-10	4.71	4.58	4.64	3.89
230421-11	5.18	5.20	5.19	5.50
230421-12	4.98	4.95	4.97	5.16
230421-13	4.80	4.83	4.82	4.15
230421-14	4.72	4.73	4.73	4.21
230421-15	4.76	4.77	4.77	4.14
230421-16	4.43	4.52	4.47	4.26
230421-17	4.93	4.88	4.90	4.33
230421-18	4.94	4.97	4.96	4.67
230421-19	4.57	4.63	4.60	4.37
230421-20	4.73	4.76	4.75	4.84
230421-21	4.91	4.87	4.89	4.19
230421-22	4.90	4.95	4.92	4.44
230421-26	4.92	4.91	4.92	4.22
230421-27	4.52	4.53	4.52	4.22

230421-28	4.78	4.76	4.77	4.07
230421-29	4.77	4.81	4.79	4.86
230421-30	4.41	4.45	4.43	3.85
230421-31	4.99	5.04	5.01	4.71
230421-32	4.46	4.32	4.39	4.04
230424-1	5.61	5.61	5.61	5.98
230424-3	6.01	5.96	5.98	5.72
230424-4	4.79	4.79	4.79	5.04
230424-6	6.60	6.57	6.58	6.47
230424-7	5.98	5.97	5.98	5.92
230424-8	5.07	5.01	5.04	4.98
230424-10	4.88	4.83	4.86	5.08
230424-11	5.77	5.80	5.79	6.40
230424-13	6.26	6.26	6.26	6.43
230424-14	6.13	6.13	6.13	6.57
230424-15	4.97	5.08	5.02	4.90
230424-16	6.00	5.98	5.99	5.54
230424-17	5.49	5.48	5.49	5.09
230424-20	5.28	5.29	5.29	6.07
230424-21	5.40	5.44	5.42	5.37
230424-22	6.25	6.26	6.25	6.20
230424-23	5.50	5.53	5.52	5.56
230424-24	4.76	4.79	4.77	4.50
230424-25	4.92	4.93	4.93	5.21
230424-26	5.90	5.93	5.92	6.07
230424-27	5.80	5.79	5.79	5.80
230424-28	5.82	5.89	5.85	6.24
230424-29	5.80	5.80	5.80	6.64
230424-30	5.06	5.00	5.03	5.09
230424-31	5.79	5.74	5.77	5.63
230424-32	6.14	6.16	6.15	6.20
230428-1	5.96	5.97	5.97	6.34
230428-2	6.03	6.00	6.01	6.07
230428-3	5.90	5.91	5.90	5.17
230428-4	5.60	5.62	5.61	5.96
230428-5	4.67	4.66	4.67	4.51
230428-6	6.13	6.14	6.14	6.10
230428-7	5.10	5.15	5.12	5
230428-8	5.02	5.00	5.01	4.81
230428-10	5.09	5.08	5.09	4.74
230428-11	4.79	4.81	4.80	5.06
230428-12	5.11	5.05	5.08	4.67
230428-13	4.59	4.70	4.65	3.94
230428-14	5.01	5.06	5.04	4.63
230428-15	4.94	4.97	4.96	5.09

230428-16	4.97	4.97	4.97	4.12
230428-17	4.93	4.93	4.93	5.12
230428-18	4.76	4.74	4.75	4.13
230428-20	5.14	5.17	5.15	4.32
230428-21	5.25	5.40	5.32	4.98
230428-22	5.28	5.29	5.29	4.48
230428-23	4.71	4.68	4.69	4.07
230428-24	5.16	5.14	5.15	4.28
230428-25	4.93	4.93	4.93	4.75
230428-26	5.32	5.29	5.31	5.47
230428-27	4.80	4.79	4.80	4.19
230428-28	4.79	4.81	4.80	4.54
230428-29	6.53	6.54	6.54	6.41
230428-30	5.71	5.73	5.72	5.37
230428-31	6.10	6.11	6.11	6.36
230428-32	5.23	5.27	5.25	4.18
230428-33	4.84	4.81	4.82	4.09
230428-34	4.97	4.94	4.96	4.58
230428-35	5.79	5.65	5.72	4.80
230428-36	5.54	5.53	5.54	5.25
230428-37	4.79	4.73	4.76	4.22
230502-5	5.93	6.00	5.96	6.30
230502-7	6.50	6.49	6.49	6.53
230502-8	6.49	6.52	6.50	5.88
230502-9	4.57	4.69	4.63	4.09
230502-10	6.50	6.48	6.49	6.24
230502-12	6.19	6.16	6.17	6.56
230502-13	6.53	6.51	6.52	6.13
230502-14	5.92	5.89	5.90	5.18
230502-17	6.52	6.53	6.52	6.56
230502-24	5.94	6.00	5.97	5.76
230502-26	5.35	5.37	5.36	5.51
230502-27	6.38	6.40	6.39	6.54
230502-28	6.12	6.12	6.12	6.01
230502-35	6.14	6.08	6.11	6.21
230502-36	6.14	6.18	6.16	6.27
TOT			344	344