

SUMMARY OF LA LETTRE DE CECALAIT, N° 40 (1ST quarter 2002)

(Translation : A. BAPTISTE, Correction : H. LAMPRELL)

Evaluation of the Bactocount IBC

(based upon the CECALAIT phase I evaluation report)

Bactocount IBC is an automatic analyser for the determination of bacteria counts in raw milk, developed by BENTLEY Instruments (USA) and marketed in France by its french subsidiary, Bentley Instruments sarl. It works using the flow cytometry principle, with an epifluorescence microscopy detection, after chemical, heat and ultrasonic treatment. CECALAIT has recently evaluated its analytical characteristics (phase I assay)

APPARATUS

It is run by a micro-computer for analyses and calibration. The sample is automatically taken and transferred into a well of the incubation wheel (with an incubation reagent). The reagent consists of a fluorochrome alkaline buffered solution, proteolytic enzyme and reaction catalyst, in order to solubilize and disperse protein, fat and somatic cells and to stain bacterial nucleic acids. The mixture is then incubated 8 mn at 50°C and part of it is injected into a hydrodynamically focused fluid stream. The bacterial cells flow one by one through a laser beam, which excites the stained cells, detected by an epifluorescence microscope objective. The fluorescent impulses of stained bacteria are discriminated, amplified by a photomultiplier, counted and converted into Individual Bacterial Cell (IBC)/ ml. A laboratory performed calibration allows the conversion of IBC / ml into CFU / ml.

EXPERIMENTAL

Using non-heated samples, the following characteristics were evaluated :

- stability
- carry-over effect
- linearity
- determination of the detection limit
- repeatability
- accuracy
- influence of milk composition

The evaluation was performed according to IDF standards 100B, 128A, 135B, 161A and to AFNOR NF V 03-110 standard.

① STABILITY

The stability was evaluated by the duplicate automatic analysis of milk sets, every 15 mn for half a day (about 20 measurement cycles), according to the actual working conditions of a milk payment laboratory.

Repeatability and reproducibility calculations were performed according to IDF 135 B.

The results of the test, performed as usual with 3 counting levels and 2 different methods of milk sample preservation showed a stability defect of the analyser, exclusively at the lower counting levels. BENTLEY Ins attributed it to the initially selected counting threshold. Therefore, they modified this setting for the further tests. Two additional tests were performed then :

- pre-test using two herd milk samples : a rich one and a poor one, stored between 0 and 2 °C
- full test using a set of 6 duplicate milk samples, ie a poor one, a medium one and a rich one, stored between 0 and 2°C, with and without addition of the azidol preservative (0.33%).

The new results showed a relative geometric standard deviation of reproducibility (GRSD_R) from 6 to about 15 %. It seems dependent on the contamination level, but independent of the method of preservation. However, at the lower contamination levels (poor milk samples), these values (about 6%) are close to the observed repeatability values (mean standard deviation of repeatability $S_r=0.023$ log). The mean standard deviation of reproducibility (S_R) is 0.043.

② CARRY-OVER

The carry-over effect was evaluated by the duplicate automatic analysis of two milk sets (rich and poor), 20 times, in the following sequence : RICH MILK – RICH MILK – POOR MILK - POOR MILK.

The test was performed using 3 different levels, with individual milks or reconstituted milks (by a mixture of microfiltration retentate and filtrate). The instrument settings had been performed by BENTLEY Ins (contamination coefficient set at 0).

The carry-over effect (T_c %) was estimated with following equation :

$$T_c\% = \left[\frac{\sum (\text{POOR1}) - \sum (\text{POOR2})}{\sum (\text{RICH2}) - \sum (\text{POOR2})} \right] \times 100$$

Under these conditions, the carry-over of the Bactocount IBC was around 0.0 to 0.05 % whatever the average contamination level of the sample. These values comply with the maximum limit of 1% usually allowed in routine methods for determination of milk composition, used for milk payment purposes. In the same context, this limit may also be used for bacterial enumeration.

③ EVALUATION OF LINEARITY

Linearity was evaluated by triplicate automatic analysis, following increasing, then decreasing levels, of a series of milks with bacterial contamination evenly distributed over the whole area of counting.

Two types of milk matrices were used :

- Milk enriched by low temperature (4 à 8 °C) incubation for 24 to 72 hours.
- recombined milk made with a mixture of microfiltration retentate, microfiltration filtrate and cream.

Whatever matrix (natural or recombined milk), the results showed a minor linearity defect of the instrument on the whole range tested. The manufacturer should apply a 3-order polynomial to the bulk signal to correct this defect. (mean linear $S_{y,x}$: 0.031 log ; 3-order polynomial S_{yx} : 0.016 log). Having thus obtained a satisfactory linearity on the whole counting area, the users could then calibrate the instrument using a simple linear equation.

④ LIMIT OF DETECTION

The detection limit of a method is the lowest value for which the user of the method can be sure that it is different from 0.

Whatever the mathematical calculation model used in order to determine the lower limit of detection, the instrumental signal was significantly different from the blank signal at a counting level as low as $0.33 \cdot 10^3$ CFU / ml.

However, the instrument's detection threshold corresponds to the precision required for routine purposes.

⑤ EVALUATION OF REPEATABILITY

The repeatability was evaluated by automatic analysis of :

- 822 (820 finally kept) herd milk samples, analysed according to IDF standard 128 (the sample racks are analysed twice in rapid succession)
- 410 (409 finally kept) samples selected among the 822 samples taken for evaluation of the accuracy, analysed in duplicate in rapid succession (repeatability setting of the instrument).

The results were first obtained in 10^3 IBC / ml (there is a precalibration of the instrument to convert IBC values into CFU equivalent values : slope : 0.5). They were then transformed into Log CFU_{Bactocount IBC} / ml and later into Log CFU / ml equivalent values using the calibration equations obtained in the course of the accuracy evaluation.

Indeed, two calibration equations had been defined according to the instrument's settings. The first tests had been performed using the initially selected counting threshold, which was later modified after the non-satisfactory first stability tests. This initial threshold, called threshold 1, corresponded to following settings : WTH = 29.3 ; HTH = 0.36*.

However, all values obtained during CECALAIT's tests were calculated again later by BENTLEY Ins, using a new threshold. This new setting had been defined in order to improve the instrument's precision when it was used in the LIAL Franche-Comté laboratory, which was one of the laboratories that performed the "phase II" evaluation. This new threshold, called threshold 2, corresponded to following settings : WTH = 0.0 ; HTH = 0.40

So, the calibration equations used are :

- with Threshold 1 : $Y = 0.8291 \times APP + 0.5406$
- with Threshold 2 : $Y = 1.0463 \times APP - 0.624$

where Y is the Log CFU/ml value, given by the reference method and X the CFU_{Bactocount IBC} given by the instrument

* *WTH* : corresponding to the width of the peak
HTH : corresponding to the height of the peak.

However, there was not enough time left to perform all the tests again. So in repeatability tests, only the tests in duplicate in rapid succession were repeated.

Tables 1 to 3 show the results. The levels correspond to french milk payment levels. See keys for tables 1 to 3 in La Lettre de CECALAIT, pages 2-3

The three tables show that the standard deviation of repeatability S_r is about 0.0445 log, *ie* the relative geometric standard deviation of repeatability is 10.8%. This value remains far below the limit recommended for milk payment purposes for bacterial counting ($S_r = 0.15$ log).

⑥ EVALUATION OF ACCURACY

Accuracy was estimated by using the residual standard deviation ($S_{y,x}$), where Y is the value given by the reference method (Log CFU / ml) and X the "CFU equivalent" given by Bactocount IBC (Log CFU_{Bactocount IBC} / ml).

The samples giving plates with too high a bacterial count, or with contaminating or spreading colonies, were discarded.

↳ Procedure

In order to obtain a representative population, with evenly distributed bacterial contamination, 410 herd milks were selected according to the results of a duplicate analysis (IDF 128) using the Bactocount IBC. They were selected among the 822 samples taken (on 9 different days, from march to june 2001) by two different interprofessional laboratories (LIAL FC -Laboratoire Interprofessionnel d'Analyses Laitières de Franche-Comté- and LDA 39 -Laboratoire Départemental d'Analyses du Jura-.

Milks were kept at 0-2 °C for 2 to 4 hours prior to analysis. Instrumental analyses were performed in consecutive duplicate, followed by a duplicate analysis using the reference method (IDF 100 B).

The analyses were performed on 9 non-consecutive days over a 4 month period, where the instrument's setting was kept unchanged. Each analytical series came from the same bulk-milk tank (24 to 48 h of tank storage), taken in duplicate at the farm and transported following the usual way for milk payment samples.

However, three samples with abnormally high repeatability values were not included in the statistical treatment for accuracy.

↳ Results

As for the evaluation of repeatability, the tests had been performed using threshold 1. But, in the same way, the values were also recalculated using threshold 2 (see ☺).

The results shown here were obtained using the best setting, ie threshold 2.

A simple linear regression was applied. It was calculated (on log transformed values) on the population of 371 milk samples (mean bacterial level : 41000 CFU/ml) and gave the following relation above 2000 (lower detection limit claimed by the manufacturer):

Threshold 2 (WTH = 0.0 ; HTH = 0.40)

Log (Reference) = 1.0463 x Log (CFU_{Bactocount IBC} / ml) - 0.6240

with mean bias to the reference values = + 0.392 & Sy,x = 0.388

The estimation of precision obtained was :

± 1.96 x 0.388 ie ± 0.760 Log CFU / ml

That means that the upper and lower limits of the 0.95 confidence interval for Y are

Log Y + 0.760 and Log Y - 0.760

However, considering only the range 10 000 to 1 000 000 CFU/ml, the residual standard deviation became : 0.335 Log. The estimation of precision then became :

± 1.96 x 0.335 soit ± 0.657 Log CFU / ml

That means that the upper and lower limits of the 0.95 confidence interval for Y are

Log Y + 0.657 and Log Y - 0.657

The instrument's precision improved when the lower level samples were discarded (below 10000 CFU / ml). This may be related to the observed linearity defect (cf ☺), affecting the lower values more.

These performances were obtained by using a simple linear regression for studying the accuracy. Considering the observed minor linearity defect, they could certainly be improved, provided that the bulk instrument signal is linear over the whole measuring range.

🕒 EVALUATION OF THE INFLUENCE OF MILK COMPOSITION

Further tests were conducted in order to study a possible influence of the composition of the milk samples on the accuracy

performance. There was no significant reduction of the residual variance. The bacterial enumeration performed by Bactocount IBC thus seems insensitive to the fat, protein or somatic cell content of milks.

CONCLUSION

The Bactocount IBC -at the time, a prototype, without temperature stabilization -, was evaluated (phase I) at CECALAIT, seeking an agreement for milk payment purposes. Stability, carry-over and repeatability have been found satisfactory.

Concerning linearity and accuracy, which are closely related, it was concluded that the linearity had to be adjusted in order to have a better idea of the accuracy performance of the instrument.

At the end of this phase, the manufacturer committed itself to setting the necessary changes in the instrument, especially regarding linearity.

That had to be done. Indeed, the instrument, since then, was used under routine conditions, in two interprofessional laboratories (LIAL FC and LDA39) for four months, for the assays corresponding to the phase II of the evaluation procedure for the agreement for milk payment purposes. This study in the two labs gave the following repeatability and accuracy results :

➤ REPEATABILITY (ALL LEVELS)

see table page 4 of la Lettre de CECALAIT

➤ ACCURACY (simple determination by Bactocount)

↳ LDA 39

(426 samples, mean bacterial level 49000 CFU/ml)

Log (Reference) = 0.7309 x Log (IBC/ ml) + 1.18

with Sy,x = 0.283 log

The estimation of precision is then :

± 1.96 x 0.283 ie ± 0.5547 Log UFC / ml

↳ LIAL FC

(498 samples, mean bacterial level 20000 UFC/ml)

Log (Reference) = 0.6422 x Log (IBC/ ml) + 1.405

with Sy,x = 0.309 log

The estimation of precision is then :

± 1.96 x 0.309 ie ± 0.605 Log UFC / ml

However, considering only levels higher than 10 000 CFU/ml, the results become :

Log (Reference) = 0.4867 x Log (IBC/ ml) + 2.286

with Sy,x = 0.264 log

The estimation of precision is then :

± 1.96 x 0.264 ie ± 0.5174 Log UFC / ml

The new tests, performed during the phase II of the evaluation of the instrument, finally validated the manufacturer's changes, especially regarding linearity and accuracy. Therefore, the instrument's calibration can be performed by a simple linear regression. Moreover, accuracy appeared better in phase II than in phase I.

Considering the whole set of results, the CST (Commission Scientifique et Technique) concluded that the analytical

performances of the instrument comply with the requirements of milk payment purposes. Bactocount IBC of Bentley Instruments can now officially be used for milk payment purposes (see French Official Journal, 3rd January 2002).

The list of abbreviations and bibliographic references are in « La Lettre de CECALAIT »

INTERESTING RECENT EU REGULATION

Regulation (EC) No 178/2002 of the European Parliament and of the Council of 28 January 2002 **laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety** (JOEC L31 of 1 february 2002)

Commission Directive 2002/23/EC of 26 February 2002 amending the Annexes to Council Directives **86/363/EEC**as regards the fixing of maximum levels for **pesticide residues in and on** foodstuffs of animal origin....(JOEC L64 of 7 march 2002

Commission Recommendation of 4 March 2002 on the reduction of the presence of dioxins, furans and PCBs in feedingstuffs and foodstuffs (JOEC L67 of 9 march 2002)

➤ also interesting

In JOEC C38 of 12 february 2002)

List of Member States' authorisations of food and food ingredients which may be treated with **ionising radiation**

&

List of approved facilities for the treatment of food and food ingredients with **ionising radiation** in the Member States

Commission Directive 2002/16/EC of 20 February 2002 on the use of certain epoxy derivatives in **materials and articles intended to come into contact with foodstuffs** (JOEC L51 of 22/02/2002)

Commission Directive 2002/17/EC of 21 February 2002 amending Directive 90/128/EEC relating to **plastic materials and articles intended to come into contact with foodstuffs** (JOEC L58 of 28 february 2002)

Official Journals of the European Communities of the last 45 days may be consulted on the Internet <http://europa.eu.int/eur-lex>

Older texts may be searched by their date on http://europa.eu.int/eur-lex/en/search/search_lif.html or consulted according to their topics on the Internet <http://europa.eu.int/eur-lex/en/lif>

INTERESTING NEW STANDARDS

ISO STANDARDS

ISO 14892:2002, february 2002 Dried skimmed milk -- Determination of vitamin D content using high-performance liquid chromatography.

IDF / ISO STANDARDS

The standards below were all elaborated by the SC5 sub-committee (milk & milk products) of ISO/TC 34 committee, where IDF and AOAC International are collaborating. From now on, ISO and IDF will jointly publish these standards, whereas AOAC International will still publish them separatly.

IDF 20-1:2001, IDF 20-2:2001, IDF 20-4:2001, IDF 20-5:2001 = (respectively) ISO 8968-1:2001, ISO 8968-2:2001, ISO 8968-4:2001, ISO 8968-5:2001

(ICS 67.100.10 : *Lait*) MILK-- Determination of nitrogen content

Part 1: Kjeldahl method

Part 2: Block-digestion method (Macro method)

Part 4: Determination of non-protein-nitrogen content

Part 5: Determination of protein-nitrogen content

IDF 60:2001 = ISO 5944:2001 (ICS 07.100.30 : *food microbiology* ; 67.100.01 : *Milk and milk products in general*) Milk and milk-based products -- Detection of coagulase-positive staphylococci -- Most probable number technique

IDF 80-1:2001 = ISO 3727-1:2001 (ICS 67.100.20 : *butter*)

Butter -- Determination of moisture, non-fat solids and fat contents

Part 1: Determination of moisture content (Reference method)

NB : Part 2: Determination of non-fat solids content (Reference method) seems already published by ISO (3727-2:2001), but not yet by IDF

➤ In the latest issue of la Lettre de CECALAIT, we already pointed out the standards below, which also issued as IDF standards. So :

EN ISO 8261 Milk and milk products -- General guidance for the preparation of test samples, initial suspensions and decimal

dilutions for microbiological examination also issued as IDF 122:2001

ISO 14156:2001 MILK AND MILK PRODUCTS. Extraction methods for lipids and liposoluble compounds also issued as IDF 172:2001

ISO 14673-1/-2/-3:2001 MILK AND MILK PRODUCTS. -- Determination of nitrate and nitrite contents also issued as IDF 14673-1 / 2 / 3 : 2001

➤ to be followed

For the first time in 40 years, FAO and WHO have begun to evaluate **Codex Alimentarius**. One of their most interesting topics is how food standards are elaborated. Official questionnaires will be circulated to Member States, but there should also be visits on field and people's interviews. The evaluation procedure is on progress until 2003 and will end in a report with recommendations for heads of FAO and WHO.

FAO : Food and Agricultural Organization

WHO : World Health Organization

Interesting reading

Besides the list of bibliographic references, other interesting reading is pointed out below.

↳ paper articles !

- an article in *Journal of Allergy & Clinical Immunology*, I: population study of food allergy in France, 2001, V. 108, n° 1, p. 133-140

- the issue 2001, volume 21, n° 4 of the journal *Science des Aliments* is all on "Nutritional Recommendations for the French Population".

- EHEDG issued a special document (n°22) on : **general criteria of hygienic conception for the safe production of dry products** (21 pages).

EHEDG = European Hygienic Engineering & Design Group

for any inquiry, please contact ASEPT, Albert AMGAR ☎
33.2.43.49.22.22 (www.asept.fr)

- the proceedings of the **3rd International Symposium on propionibacteria**, held in Zürich (Switzerland) from 8th to 11th july 2001, issued in LAIT, 2002, V. 82, n° 1.

↳ Internet

- a site concerning the "**protected denomination of origin**" and neighbouring products : <http://www.origin-food.org>

- all about microbiology on <http://www.microbiology-direct.com>

- le virtual dictionary of the International Union of Pure and Applied Chemistry (IUPAC)

<http://chemsoc.org/chembytes/goldbook/index.htm>

- "Questions and answers" from the Global Forum for food safety responsables, held in Marrakech, from 28th to 30th january 2002, under behalf of WHO

http://www.who.int/fsf/Global_forum_Q&A_EN.pdf

AFNOR VALIDATION

↳ EXTENSION OF VALIDATION

The validation of following method was extended to all food and feed products : **LUMIPROBE 24h**, manufactured by EURALAM, for the quick detection of *Salmonella*, (formerly validated only for egg and egg products).

↳ RENEWALS

The validation of following methods was renewed :

- **Rapid E. coli 2**, a medium manufactured by BIO-RAD for the enumeration of β -glucuronidase positive *E. coli*, until november 19th 2005, for all human foodstuffs.

- **Probelia Listeria monocytogenes** also manufactured by BIO-RAD for the detection of *L. monocytogenes* until january 21st 2006, for all human foodstuffs.

↳ END OF VALIDATION

The manufacturer did not ask for a renewal of **PENZYM 100**, a test for the specific detection of β -lactams.

↳ IMPORTANT DECISION OF THE AFNOR COMMISSION OF VALIDATION

At the beginning of year 2002, the AFNOR Commission of validation decided that the **confirmation of all presumptive positive samples** should become compulsory for all AFNOR validated test kits for the detection of pathogens in food.

Confirmation should be performed by the usual biochemical tests described in standards and should use the same enrichment broth as the one used to perform the test kit method.

The manufacturers of the test kits previously validated without confirmation will have to change their instruction sheets by June 2002 and specify that the **confirmation of all presumptive positive samples should be systematically performed**.

In the same way, AFNOR will amend the previously issued papers concerning validated test kits.