



2011 Annual Report of the European Union Reference Laboratory for Milk and Milk Products

Version 0 – 31/03/2012

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0 INTRODUCTION

The Maisons-Alfort Laboratory for Food Safety of Anses (French agency for food, environmental and occupational health safety) –formerly Afssa-Lerqap- has undertaken, as European Union Reference Laboratory (EURL) for milk & milk products (EURL MMP, formerly CRL MMP), the following works in 2011 according in particular to (a) the actions planned at the 12th Workshop of the National Reference Laboratories (NRLs) (28&29 May 2010), and (b) the work programme defined in Annex I of the Framework Partnership Agreement between EC/DG SANCO and the EU-RL for the period 2006-2011.

These actions are part of the current mandate of the EURL MMP, restricted to the control of raw and heat-treated liquid milk (total flora, somatic cells count, phosphatase activity), as well as cheeses for phosphatase, in the frame of the Regulation 853/2004 *laying down specific hygiene rules for food of animal origin*.

The Annex III, Section IX of Regulation 853/2004 is dedicated to raw milk and dairy products:

- Microbiological criteria on total flora at 30°C and on somatic cells count are fixed:
 - o At the level of raw milk production & collection: for raw cow's milk and raw milk from other species milk (Chapter I, clauses I & III);
 - o At the level of preparing dairy products (Chapter II, clause III-criteria for the use of raw cow's milk for further processing).
- Phosphatase activity:
 - o At the level of raw milk production (Chapter I, clause I.3): a reference is made to a negative phosphatase test to characterize the heat-treatment to be applied to raw cow's or buffalo's milk coming from animals not meeting certain requirements on brucellosis or tuberculosis.
 - o At the level of heat treatment of raw milk or dairy products (Chapter II, clause II): the food business operators shall ensure that the heat-treatment satisfies the requirements of Regulation 852/2004, Annex II, Chapter XI.

The EURL has provided in particular a support to the NRLs for the implementation of:

- the Regulation 853/2004;
- the derived Regulation 2074/2005 modified by Regulation 1664/2006, defining amongst other the testing methods for raw milk and heat-treated milk to be used by competent authorities and food business operators:
 - o to check compliance with the limits for total flora and somatic cells count laid down in Regulation 853/2004, Annex III/Section IX/Chapter I/Part III,
 - o to ensure appropriate application of a pasteurisation process to dairy products, as referred to in Regulation 853/2004, Annex III/Section IX/Chapter II/Part II.

NB: in brackets under each item, the scheduled duration of the action is indicated: either annual (limited to 2011), either multi-annual (on-going programme on several years).

1 HYGIENE OF RAW MILK

Frame: The Regulation 2074/2005 modified by Regulation 1664/2006 prescribes the reference method for total flora enumeration at 30°C, Standard EN ISO 4833, and the reference method for somatic cells count, Standard EN ISO 13366-1, as well as conditions for the use of alternative methods.

1.1 INTER-LABORATORY PROFICIENCY TESTING FOR THE NRLS

1.1.1 STUDY OF SAMPLE TYPES USED FOR INTER-LABORATORY TRIALS ON TOTAL FLORA IN RAW GOAT'S MILK

(multi-annual)

In the frame of the organization of the 2011 proficiency testing (PT) trial dedicated to the enumeration of total flora (TF) in raw goat's milk, the EURL MMP (Unit EDB) had begun in 2010 an investigation study on the homogeneity and on the stability of the samples to be used for this trial to find a way to stabilize sufficiently the TF contamination of raw goat's milk. The chemical stabilizer chosen must allow the bacteria to grow on plates after the dilution steps.

Two different studies were performed in 2011 by using a chemical agent. Samples of raw goat's milk were stored at 12°C and 3 levels of inoculation (i.e. low, medium and high levels) were tested. For the homogeneity study, TF enumeration was performed in duplicate at 0 day, from 10 samples of each level. For the stability study, TF enumeration was performed in duplicate each day during 11 days, from 3 samples of each level.

The EURL is currently preparing the report of the study which will be soon dispatched.

1.1.2 STUDY OF SAMPLE TYPES USED FOR INTER-LABORATORY TRIALS ON SOMATIC CELLS IN RAW COW'S MILK

(multi-annual)

In the frame of the organization of the 2012 PT trial dedicated to the counting of somatic cells (SC) in raw cow's milk, the EURL MMP (Unit EDB) began in 2011 an investigation study on the homogeneity and on the stability of the samples to be used for this trial.

Two different studies were performed in 2011 by using a chemical agent. Samples of raw cow's milk were stored at 8°C. Three levels of inoculation (i.e. low, medium and high levels) were tested. For the homogeneity study, SC counting was performed in duplicate at 0 day, from 10 samples of each level. For the stability study, SC counting was performed in duplicate each day during 13 days, from 3 samples of each level.

1.1.3 ENUMERATION OF TOTAL FLORA AT 30°C IN RAW GOAT'S MILK

The inter-laboratory (PT) trials aim is to evaluate the ability of the NRLs to apply correctly the reference methods prescribed by European Regulation 2074/2005 modified.

The EURL MMP (EDB Unit) conducted in November 2011 a PT trial on TF enumeration at 30°C in raw goat's milk. 31 NRLs participated to this trial. The Standard EN ISO 4833 (total plate count) method was used to enumerate the total flora.

The EURL prepared and dispatched in advance (Week 45) the instructions and the test report for the trial. The EURL prepared samples (6 samples of raw goat's milk, with a different given TF level) and dispatched them in November (Week 46) to the participating laboratories.

The EURL has collected the results and is currently preparing the PT trial report which will be soon dispatched.

1.2 ANALYTICAL DEVELOPMENT

(multi-annual)

1.2.1 DETERMINATION OF TOTAL FLORA AT 30°C AND SOMATIC CELLS IN RAW MILK BY AN INSTRUMENTAL METHOD

1.2.1.1 DETERMINATION OF TOTAL FLORA AT 30°C IN RAW COW'S MILK

The EURL MMP (Unit EDB) had launched an experimental study in 2007 using a flow cytometer (Bactocount, Bentley) as an alternative method for the enumeration of bacterial TF and SC in raw cow's milk, in order to investigate the correlation relationship between this method and the respective reference methods, especially the influence of production factors of raw milk (mainly variation in breeds, period of lactation, type of feeding) on this conversion relationship.

The EURL has dispatched its Technical Report "Factors influencing the conversion relationship between the reference method for enumeration of total flora at 30°C in raw cow's milk and an alternative method based on flow cytometry" by Circular Letter dated 16 January 2012.

Furthermore, in 2011, the EURL has developed an experimental design for studying the influence of other factors (such as environment of animals, storage of milk) on the conversion equation for cow's milk. This 2nd phase of the study will begin in 2012.

In addition, once a month, 9 samples were analysed for SC counting by the reference method, the Standard EN ISO 13366-1, and by the alternative method based on flow cytometry.

1.2.1.2 DETERMINATION OF TOTAL FLORA AT 30°C IN RAW GOAT'S MILK

The EURL MMP (Unit EDB) had launched an experimental study in 2010 using a flow cytometer (Bactocount, Bentley) as an alternative method for the enumeration of bacterial TF in raw goat's milk, in order to investigate the correlation relationship between this method and the respective reference methods, especially the influence of production factors of raw milk (mainly variation in breeds, period of lactation, type of feeding) on this conversion relationship.

This experimental study has been continued in 2011. 129 samples have been analyzed in 2010 and 2011. Samples are received from 15 different producers of 3 districts. Milk samples are collected from 3 different goat's breed.

1.2.1.3 DETERMINATION OF TOTAL FLORA AT 30°C IN COW'S COLOSTRUM

Frame: In the frame of Regulation 853/2004 modified, national hygienic requirements are referred to for colostrums.

The EURL MMP (EDB Unit) had undertaken in 2010 an enquiry to the NRLs to collect existing national hygienic requirements/microbiological criteria on cows' colostrums.

The EURL has presented at the 2011 workshop a synthesis of the outcome of this enquiry.: no national criterion has been reported in most of the 21 Member States (respondents) and for the ones having a criterion the same levels have been chosen as for raw cow's milk. At the workshop, it was decided that the EURL would launch a new enquiry on total flora and somatic cells count levels (or other hygienic/pathogenic bacteria), as well as antibiotic residues found in raw colostrums for direct human consumption, in parallel with a review of the literature.

The EURL has prepared a written report on this survey, which was dispatched to DG SANCO and NRLs by Circular Letter dated 14/02/2012.

1.3 COORDINATION OF THE NRL ON DETERMINATION OF TOTAL FLORA

(multi-annual)

The survey on the determination of total bacterial count in raw milk in EU Member States dispatched in 2006 had been recirculated to the NRLs in 2010, whose outcome had been presented at the 2010 workshop dedicated to total flora in raw milk. The EURL has prepared a report of this enquiry and dispatched it to DG SANCO and NRLs by Circular Letter dated 11/04/2011.

2.4 STANDARDISATION ON VALIDATION OF ROUTINE METHODS FOR TOTAL FLORA IN RAW MILK

Criteria for the validation of instrumental (epifluorescent) methods for the determination of total flora in raw milk

According to the European regulation 2074/2005 modified by Regulation 1664/2006, the use of alternative methods to the reference method EN ISO 4833 is possible if they are validated against the reference method in accordance with the protocol of the Standard EN ISO 16140 or other similar internationally accepted protocols.

At the 2010 workshop dedicated to TF in raw milk, it has been decided that alternative methods for TF in raw milk shall be fully validated within 2 years if they are used in the frame of Regulation 2074/2005 modified.

Up to now, no real complete validation study according to EN ISO 16140 has been performed on alternative methods for TF in raw milk, in particular lacking of an inter-laboratory validation study.

At the 2010 workshop, it had been decided that a summary of validation criteria combining the draft revision of IDF 161 (ISO/DIS 16297) and EN ISO 16140 would be prepared by the EURL MMP.

The EURL prepared this document on validation criteria of instrumental (epifluorescent) methods for the determination of total flora in raw milk, in collaboration with the NRLs: a first draft has been written and dispatched to the NRLs by Circular Letter dated 22/04/2011. The draft was also sent for consultation to the IDF/ISO Project Group S07 (in charge of the revision of the Standard IDF 161A).

Based on comments received, the EURL prepared a final version of this document, sent to DG SANCO and the NRLs by Circular Letter dated 21/12/2011. The EURL sent this document also to the validation/ certification bodies (AFNOR Certification, MicroVal and NordVal), which are to base their validation studies on it.

1.5 DEVELOPMENT OF CERTIFIED REFERENCE MATERIALS FOR SOMATIC CELLS COUNT IN RAW MILK

Given the deficiencies of the reference microscopic method for SC counting in raw milk (lack of reproducibility) and the limited number of laboratories using it, it is of utmost importance to develop Certified Reference Materials, which are not currently available.

In 2011, the EURL MMP has reiterated its request to DG SANCO for supporting the need that JRC (Geel, Belgium) develops, in cooperation with the EURL, certified reference materials for somatic cells in milk.

1.6 DEVELOPMENT OF A REFERENCE SYSTEM FOR SOMATIC CELLS COUNT IN RAW MILK

IDF/ISO has initiated the setting-up of a reference system for SCC in raw milk, given the deficiencies of the microscopic reference method to provide reference values comparable between different laboratories. It is intended that this reference system, in addition to the reference method, would take account of reference materials and of instrumental methods used in routine. A network of expert laboratories is intended to be settled, to define assigned values associated to reference materials used for the calibration of instrumental methods.

The EURL has further participated to the IDF/ICAR working group in charge of developing this reference system as to envisage how it could be beneficial to the EURL/NRLs own works, to implement the requirements of Regulation 853/2004 concerning SC. It has kept informed the NRLs of progress made during the 2011 workshop.

2 DETERMINATION OF ALKALINE PHOSPHATASE ACTIVITY

Frame: The European Regulation 2074/2005 modified by Regulation 1664/2006 defines the reference method, the Standard EN ISO 11816-1, the legal limit for negativity of the test for alkaline phosphatase (AP) activity for correctly pasteurised cow's milk (350 mU/l) and conditions to use alternative methods.

2.1 INTERNATIONAL VALIDATION TRIAL AND, IN PARALLEL, INTER-LABORATORY PROFICIENCY TESTING FOR THE NRL ON AP DETERMINATION IN CHEESE

Following discussion and advice of the international joint group of experts IDF/ISO, the EURL MMP (Team CAT-AP) has modified the design of validation and proficiency tests on AP determination in cheese set by EURL, in collaboration with the NRLs during the 2011 workshop. The target of the modification was to include a preliminary study regarding the impact of sample preparation on the performances of the method.

The international experts (including EURL and a number of NRL representatives) deemed important to evaluate the contribution of the clause on preparation of the test sample on the overall variability of the results obtained with the prescribed protocol, before moving to a full validation study, and thereafter to a proficiency test. Consequently, it was decided to proceed to a two-part preliminary validation study, with a limited number of laboratories, both on cheese samples as commercially available and on the same samples prepared by EURL according to the clause on preparation of the test sample of the revised draft standard. Consequently the action was postponed to 2012.

2.2 ANALYTICAL DEVELOPMENT & STUDIES SUPPORTING EUROPEAN LEGISLATION

(multi-annual)

In 2011, the EURL MMP (Team CAT-AP) has conducted the following activities. It should be noted that the scientist upon EURL MMP contract, Caroline VIGNAUD, left the laboratory in July 2011 and could not be replaced until the end of 2011, thus all the activities scheduled in the 2011 work programme could not be conducted.

2.2.1 STUDY OF THE IMPACT OF THE BETWEEN-INSTRUMENTS VARIABILITY TO THE OVERALL VARIATION OF RESULTS OBTAINED BY DIFFERENT FLUOROPHOS UNITS

A study on the three different Fluorophos instruments in service within EURL was conducted so as to evaluate the impact of the instruments themselves on the variation of the results obtained on the same test samples, analysed with the same reagents and under overall identical conditions.

Both cow's milk -whole, skimmed and semi-skimmed milk- and goat's milk -semi-skim- having different levels of AP activity, were submitted to the study. The method applied was the reference method EN ISO 11816-1 "Milk and milk products - determination of alkaline

phosphatase activity – Part 1: Fluorimetric method for milk and milk-based drinks” and all samples were analysed in duplicate.

Differences between all results obtained on the three fluorimeter equipments (expressed as the mean of two duplicate determinations) are lower than the repeatability limit of the method, thus confirming that the impact of individual instrument variability is negligible and does not impact the results obtained. Instrument number 3 showed a very slight trend to overestimate results compared to the two other equipments but the gap is still below the repeatability value.

The EURL is finalizing a report prior to circulation to NRLs.

2.2.2 DETERMINATION OF THE PHOSPHATASE ACTIVITY IN OTHER THAN COW'S MILK

The purpose of this work is to support DG SANCO in prescribing legal limits of AP activity in milk from different species, other than cow's milk.

2.2.2.1 EWE'S MILK

The EURL has not been able to deal with this action by lack of human resources.

2.2.2.2 CAMEL'S MILK

A project to characterize the heat-treatment of camel's milk has been developed within a technical collaboration between the Central Veterinary Research Laboratory of Dubai and the German NRL.

DG SANCO had scheduled that the EURL would be regularly advised on progress of the work but there has been no feed-back at all during 2011.

2.2.3 REVISION OF THE INTERNATIONAL STANDARD EN ISO 11816/IDF 155 (PARTS 1 & 2)

2.2.3.1 ISO 11816-1/IDF 155-1 (MILK)

The EURL MMP (Marina NICOLAS) has very actively participated to the revision of part 1 (milk) of the ISO/IDF International Standard particularly in regard to the harmonization of parts 1 & 2 of the standard (milk and cheese respectively) and to the expression of the precision figures of the standards.

2.2.3.2 ISO 11816-2/IDF 155-2 (CHEESE)

The standardization procedure followed the normal stages. In 2011, comments were received from Italy, Germany and Norway. After bilateral e-mail exchange with the experts having formulated remarks, the method was updated and accepted as the basis for international validation.

2.2.3.3 COORDINATION OF THE SURVEYS CONDUCTED BY NRL ON CHEESE MADE FROM PASTEURISED COW'S MILK

The EURL has disseminated information to NRLs upon request (NL& FI) about the types of cheese to analyze, number of samples to test within each variety, total sample size and the impact of seasonal variability.

2.2.4 COMPARISON OF THE CHEMILUMINESCENT/FLUORIMETRIC METHODS

The EURL completed the study and put into practice the approach of the accuracy profile (acceptability limits) to assess the equivalence of the chemiluminescent method versus the official fluorimetric method. Comparison dealt with cow's whole milk spiked at 3 different levels and analysed on 4 different days. Samples were analysed in duplicate with the fluorimetric method and in triplicate with the chemiluminescent method.

The statistical evaluation of the results showed that the 2 methods under study were not equivalent and that results obtained with each method were not correlated by a linear regression so that no conversion factor between the 2 was possible. Thus the chemiluminescent method cannot be used as alternative to the fluorimetric method, in the frame of Regulation 2074/2005 modified.

2.2.5 HEAT TRACERS OTHER THAN AP, DEVELOPMENT OF ANALYTICAL PROTOCOLS

The EURL made a preliminary scrutinization of a method introduced by the FDA expert on AP, Dr George ZIOBRO, on gamma-glutamyl transpeptidase which may be an interesting option for those species where AP is not applicable as a biotracer of pasteurisation.

2.2.6 REACTIVATED PHOSPHATASE

No progress on this topic.

3 ASSISTANCE TO THE NRL

In 2011, no NRL has requested to be trained by the EURL.

4 NRLS WORKSHOP

The EURL has organised the 14th NRLs Workshop, on 2&3 May 2011 in Maisons-Alfort. It was a workshop of general scope, which enabled:

- to give an update on works undertaken by the EURL since the last general workshop of 2009, and in particular further to the 2010 workshop dedicated to total flora;
- to envisage the work programme for the following years.

The report was dispatched to DG SANCO and NRLs by Circular Letter dated 13/07/2011.

5 TECHNICAL AND SCIENTIFIC ASSISTANCE TO THE EUROPEAN COMMISSION

(multi-annual)

5.1 DG SANCO ACTIVITIES

Upon request of the services of DG SANCO in charge of food hygiene: participation to the bilateral Australian visit to Europe on hygiene of raw milk cheeses: presentation of Bertrand LOMBARD on safety of raw milk cheeses (Brussels, 27 June 2011).

5.2 PARTICIPATION TO ISO/IDF STANDARDIZATION WORKS

On behalf of DG SANCO (and official nomination as EC representative to CEN/ISO meetings), participation to:

1. The IDF/ISO works on the analytical methods specific to the analysis of raw milk:
 - somatic cells count: reference and alternative methods,
 - total flora: alternative methods,
 - phosphatase test: reference and alternative methods, and other pasteurisation tracers.
2. The 2011 IDF/ISO Analytical Week (Lyon, France, 23-27 May 2011): participation of Marina NICOLAS, Véronique DEPERROIS & Bertrand LOMBARD to the meetings of the different groups dealing with the topics mentioned above.

5.3 VISIT TO NRLS

In 2011, Adrien ASSERE, co-manager of the EURL *Lm* visited Evira (FI-NRL) on 09/11/2011, the Veterinary and Food Laboratory (EE-NRL) on 14/11/2011, Bior (LV) on 15/11/2011 and the National Veterinary Laboratory (LT) on 16/11/2011.

6 PUBLICATIONS

6.1 REPORTS

Report on the determination of total bacterial count in the MSs (2011) EURL for milk and milk products (Unit EDB)

Report on validation criteria of instrumental (epifluorescent) methods for the determination of total flora in raw milk (2011) EURL for milk and milk products (Unit EDB)

Report on the 2010 inter-laboratory proficiency testing trial on somatic cells count in raw milk by the reference method, Standard EN ISO 13366-1 (2011) EURL for milk and milk products (Unit EDB)

Factors influencing the conversion relationship between the reference method for enumeration of total flora at 30°C in raw cow's milk and an alternative method based on flow cytometry (2012) EURL for milk and milk products (Unit EDB)

National hygienic requirements criteria on cow's colostrums: outcome of an enquiry to the National Reference Laboratories for Milk & Milk Products (2012) EURL for milk and milk products (Unit EDB)

6.2 ORAL PRESENTATIONS

Deperrois V., Participation à la semaine analytique de la FIL "Fédération internationale de Laiterie", Communications au groupe de travail S07, "Evaluation des méthodes alternatives", on « Criteria for the validation of alternative methods for the determination of total flora in raw milk » and «Comments on the draft revision of IDF 161 Standard (ISO/DIS 16297) » Lyon, 23-24 May 2011.

Marina NICOLAS : Insights on alkaline phosphatase and work in progress at the national, European and international level, Analytical Commission of the French National Interprofessional Centre, 22 February 2011.

Marina NICOLAS: Determination of Alkaline Phosphatase activity as a tool to characterize cheeses made from pasteurized cow milk - Prospects for the establishment of an EU regulatory threshold for cow-milk pasteurized cheese,

IDF World Dairy Summit 2011, Conference on Innovative Analytical Strategies, Parma (Italy), 17 October 2011

6.3 POSTERS

Cauquil A., Guillier L., Bouchez P., Soudrie N., Maladen V., Pierru S., Asséré A., Lombard B., Deperrois V. (2011) Étude de la relation de conversion entre le Bactocount IBCm et la méthode de référence pour la détermination de la flore totale du lait cru. Colloque SFM "Les

applications de la cytométrie en flux en microbiologie", Institut Pasteur, Paris (France), 28 février.

Deperrois V., Khelil S., Chagnot C., Miled R., Meheut T. (2011) Optimization of PCR-DGGE for the bacterial analysis of dairy products. 4th Congress of European Microbiologists, Genève, Suisse, 26 - 30 juin 2011.

Deperrois V., Khelil S., Chagnot C., Miled R., Meheut T. (2011) rpoB gene used as an alternative to V3 gene for the identification of the bacterial species of milk and dairy products. Sixteenth Conference on Food Microbiology (BSFM), Bruxelles, 22-23 septembre 2011.

Cauquil A., Guillier L., Bouchez P., Soudrie N., Maladen V., Pierru S., Asséré A., Lombard B., Deperrois V. (2011) Study of the correlation between the Bactocount and the reference method for enumeration of total flora at 30°C in raw's cow milk. Sixteenth Conference on Food Microbiology (BSFM), Bruxelles, 22-23 septembre 2011.

Marina NICOLAS₂ Communications in the frame of the IDF/ISO Project Groups P06 "Determination of the Alkaline Phosphatase activity in cheese " and P13 "Phosphatase activity in cheese made from cow pasteurized milk": "Last update of the draft revised method on AP determination in cheese" & « AP activity in French cheeses ; soft cheeses with washed rind and semi-hard cheeses from pasteurized cow milk" (respectively), Lyon, 24 and 26 May.

6.4 PUBLICATIONS FOR PROFESSIONALS

Marina NICOLAS: Successful harmonization of analytical methods at the international level, chapter on « Alkaline Phosphatase : divergent method approaches and regulatory limits to harmonize », Revue Laitière Française, May 2011.