R & D DAIRY ANALYSIS METHODS
ENUMERATION OF BUTYRIC ACID BACTERIA SPORES IN MILK: APPLICATION TO MICRORESPIROMETRY

At the beginning of 2001, a new organisation scheme in R&D for dairy analysis methods saw the light, with, at a national level, the creation of a coordination body, the Analytical Dairy Research Orientation Committee or CORAL. Put into place with a strong implication of CECALAIT, it is destined to develop methods adapted to the dairy laboratories needs.

The first R&D project, being carried out at present, in the context of CORAL, is to find an alternative to the official CNERNA method, which presents important inconveniences in terms of congestion, cost, difficulty, waste and response time. The retained solution resides in a reduction of the volumes used. This is made possible due to an automated apparatus able to carry out measurements on 96 well micro-titration plates, the Manovolumate Nano 95, invented by Bruno Verdier (CNRS) and manufactured by AISSOR.

The development programme should be finished by the end of 2004.

A NEW R&D ORGANISATION SCHEME

In 2000-2001, the construction of a new building allowed CECALAIT – in parallel with an organisation and functioning in appropriation with the requirements for accreditation – to put into place a new activity centred around R&D in dairy analysis.

CECALAIT was assigned by the dairy interprofession to put into place and conduct meetings and work destined to guarantee, at different levels, a good coordination of the actions carried out.

At a local level, the creation in Poligny of the analysis methods R&D group INRA-CECALAIT (CRD) allows the necessary exchanges between INRA and CECALAIT for joint consideration on R&D projects that may be proposed or developped. This, because of the historical link that exists between the two organisations.

As a matter of interest, since the sixties, research at INRA serves analytical needs of the dairy industry. As for CECALAIT, it took over and developped the service and control activities put into place by INRA before 1990.

At a national level, the dairy industry has provided itself with an authority entitled “Analytical Dairy Research Orientation Committee’’ (CORAL). The different people concerned, the users or those who benefit from the development of analytical methods, are represented.

The system started functioning in 2001, with the set up of a first method development project, which formed a test phase for the new organisation.

This project concerns the development of a method for the estimation of the concentration of butyric acid bacteria spores in milk, susceptible to bring about solutions to the actual limits and weaknesses of the offical CNERNA method (used in interprofessional dairy laboratories). This method, entitled "Recommendations for the estimation of the contamination of milk with *Clostridia* spores by the liquid medium culture method", is based on the estimation of the most probable number (MPN) and applied in milk payment cases.

The project emerged from a need expressed on numerous occasions by the interprofessional laboratories, resurfacing by way of the CNIEL and CECALAIT’s scientific committee.

A study project was prepared by CECALAIT’s R&D department and was proposed to the CORAL after having received a favorable opinion from the CRD, according to the local organisation of a pre-estalished meeting.

Following a feasability study requested by the CORAL, the project received its backing. Since then, the project is being developped in the context of a multi-partnership convention grouping together:

- supervision of the work and financing: the CNIEL.
- the manufacturing college: the CNRS, the companies AISSOR and R-Biopharm France
- the experimentation section: CECALAIT, LIAL MC, ITFF, CEDILAC.

The project is carried out under the supervision of CECALAIT’s R&D department (O. LERAY) and a running committee in which all the partners participate.

THE SEARCH FOR AN ALTERNATIVE TO THE OFFICIAL CNERNA METHOD

Butyric acid bacteria spores are a form of survival for these bacteria, appearing under difficult environmental conditions. As the spores are not destroyed by pasteurization, they represent a major risk in the production

of semi-hard cheeses. They are involved in swelling and bursting of cheese rounds; they are also involved in marked sensory defects. In every case, they bring about a partial or total loss in market value.

Butyric acid bacteria spores in milk originate essentially from contaminations on the farm (earth, faeces, silage). So, in order to limit contamination within the herd, technical monitoring plans were introduced, taking into account the load in butyric acid bacteria spores, in milk payment analysis to producers.

The method adopted is the CNERNA method developed by J-L BRUGERE in 1986 and published in the French Republic Official Journal.

The method is based upon the capacity of milk contaminating butyric acid bacteria spores to grow under anaerobic conditions and to produce gas following heat treatment at 75°C for 10 minutes (to avoid taking into account vegetative flora and de-oxygenize the medium).

To give the method an appropriate detection limit, the lower level of spores looked for renders necessary the use of much greater test volumes than usual in microbiology. Thus, growth in the global volume taken is it rendered possible by multiplication of the presence/absence measurements at different milk dilutions. This allows, thanks to statistical theory, to establish the most probable number (MPN) of butyric acid bacteria spores from a number of positives for each dilution (caracteristic number). The whole being designated as the MPN method.

Such as it is currently used and per dosage, the CNERNA method uses:

- Ten 20 ml test-tubes each containing 10 ml of medium,
- of which 5 test-tubes contain 1 ml milk for a dilution 0 *(no dilution)*
- and 5 other test-tubes contain 0.1 ml milk for a dilution to one tenth *(dilution-1)*
- the total volume of 5.5 ml determines a minimal estimation threshold of 180 spores / l.

The incubation period under anaerobic conditions during which germination and growth of the butyric bacteria occurs is fixed at 7 days.

A parafin plug directly in contact with the medium prevents penetration of oxygen into the medium and serves as an indicator in case significant quantities of gas are produced, that is to say quantities capable of pushing the plug up by at least 1 cm.

The positive tubes for each dilution are counted by the operators who note the characteristic number (CN) on the computer. The machine will then establish the correspondance with the most probable number (MPN), by comparison with statistical tables.

OBJECTIVES OF THE PROJECT

The objectives aimed at consist in:

1- the reduction of the inconveniences of the CNERNA method:

■ congestion: important laboratoty space laboratoire, linked to equipment size (water bath, ovens and warm rooms, results reading room) is necessary.
■ cost of consumables: test-tubes, BBMB culture medium, large quantities of parafin.
■ Cost of waste disposal: treatment and recycling.
■ Cost of staff: limited automatization, absent for acquisition and entering of results.
■ Tediousness: olfactory nuisance, visual reading and entering of results manually.

If such is the case:

■ Lengthy response time (7 day incubation period)

2 - and, by means of minimum constraint, to obtenir an alternative method:

■ characteristics equivalent to the official method (threshold, statistical precision, repeatability, specificty and accuracy or concordance with the official method, analytical rate), and
■ an automatization that is compatible with the needs of modern laboratories and quality assurance (acquisition and management of computerised results, traceability).

THE CHOICE OF THE MEANS

An obvious fact: the scale reduction of the method (obliged reduction in volumes used) brought about a solution to most of the evoked inconveniences. However, the volume of the test sample could not be modified without touching the threshold of the method and therefore constituted a limit that could not be bypassed.

Furthermore, the risk of human error becoming too important with small volumes, in so much that measurement is still possible, a sure and automated means of detection of gas production should replace visual enumeration by operators.

In the eighties, the CNRS had successfully resolved the difficult problem of the measurment of weak changes in the volumes of gas and developed manovolumetric measurement modules, results which have been patented. It is only in the nineties that applications were proposed in the dairy sector and that, notably, an automated apparatus able to carry out measurments on a 96 well (8x12) microtitration plate, the Manovolumate Nano 95, was developed jointly by the CNRS (UMR 7625) – and by the company AISSOR.

The basis of the start of the project is the result therefore of the association of the objective to reduce the size of the method with the existence of a material susceptible to give the detection hoped for.

Moreover, a better sensitivity of the measuring system and a much lower congestion allowed an opening for the evolution of the MPN method, for example towards anticipated detection (< 7 days) or a reduction in the actual estimation threshold level (< 180 spores/l) with the implementation of more milk.

The first tests began in autumn 2002 and technical adaptations have been in process since then, as much on the measurement module, the Nano 95, as on elements of the method prior to measurement.

BASIS AND INITIAL CHOICES

In general, the objective is to stay as close as possible to the official method and to make any modifications only if strictly necessary to adapt the method to a reduction in size. In effect, the object of the study consisted in establishing the conditions of concordance/discordance with the official method which result solely from the reduction in size.

If the use of smaller volumes opens the way to a possible increase in precision and decrease in the MPN estimation threshold, by playing on the number of repetitions (tubes=wells) and the dilutions – this is not part of the study and could be the object of subsequent developments.

The option was therefore taken to reduce the volume (milk and medium) so as to end up with a total volume not exceeding 1100µl, which would render possible the use of mircotitration plates with a well volume of 1200µl (maximum volume authorised due to the geometry of the apparatus with the present layout).

At the same time, the concentration of the nutritive medium (BBMB) supply was adjusted to allow germination and gas production in accordance with the manovolumetric mode of detection and the results of the CNERNA method.

STAGES OF THE ALTERNATIVE METHOD

■ Preparation of concentrated sterile medium (pH and concentration requiremements)
■ Distribution on the microtitration plates
■ Distribution of inoculated milk samples: 1 ml test sample only for the 0 dilution and 100µl test sample and 900µl UHT milk (without spores) for the -1dilution.
■ Heating for 10 minutes at 75°C with
■ the plate tightly closed and anaerobic conditions.
■ Cooling and incubation for 7 days at 37°C.
■ Preparation of the plate for analysis.
■ Manovolumetric measurement with the Nano95, registration, edition.

THE APPARATUS

The Manovolumate Nano 95

Patent CNRS – Inventor Bruno Verdier
Patent AISSOR (Metz) - Manufacturer Jean-Pascal Urban

Once the well fastner membrane is pierced directly above each well, the microtitration plates are introduced into the Nano 95 and the measurement cycle is managed automatically until obtention of the results. Each well opening is placed opposite a state capturer (capillary system in which a column of manovolumetric liquid moves according to the variations in the amount of gas in the chamber (well)). The movement of the meniscus at the liquid/gas interface is then registered and interpreted.

As from now, the measuring apparatus allows:

■ the registration of the gas release kinetics for each of the 96 wells,
■ the automatic interpretation in terms of characteristic number and the corresponding most probable number, traceability of the results and the possibility of data transfer from computer to computer.

EVOLUTION OF THE PROJECT

As from the start, the project has seen a significant evolution in the equipments initially planned, notably in response to problems posed by the importance of the

volumes of gas released. The CNRS and AISSOR have thus developed original solutions and appreciably modified the original Manovolumate Nano 96 by adapting the plate conveying system, the sensitivity and the parameters of the apparatus resulting in, at the present time, a unity ready to undergo the first test phase in routine conditions.

Several stages are forseen for the overall study:

■ Phase 0 – Initial test phase (pre-validation)

Allowing the necessary adjustments to the apparatus and to the method and the verification of the good working order of the measurement system before moving on to the next stage. This phase, in process during the writing of this article, is taking place dividedly between firstly the LIAL MC at Aurillac then CECALAIT.

■ Phase 1 – Study of the method

Scheduled in CECALAIT's laboratory, this phase will allow the characteristics of the new method to be established (specificty, linearity, sensitivity) and comparison with the CNERNA method (repeatability and accuracy/concordance).

■ Phase 2 – Evaluation under routine conditions

The method will be tested for repeatability and concordance with the offical method with a large number of milk samples from producers so as to acquire robust statistical information within the context of routine analyses in an interprofessional laboratory. The LIAL MC will assure the running of this phase which will equally allow the evaluation of other characteristics of econmical nature (analytical rate, material robustness, servicing, etc.) Phase 2 will be, in principal, renewed and the results confirmed in a second interprofessional laboratory.

The project should be finished by the end of 2004.

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Abbreviations

BBMB: Bryant-Bukey modified Bergère culture medium
CN: characteristic number
MPN: Most Probable Number

Acronyms

CEDILAC : Compagnie Européenne de Diffusion de Produits Lactés
CNERNA : Centre National d'Etudes et de Recommandations sur la Nutrition et l'Alimentation
CNIEL : Centre National Interprofessionnel de l'Economie Laitière
CNRS : Centre National de la Recherche Scientifique
CRD : Cellule Recherche et Développement Méthodes d'Analyses
CORAL : Comité d'Orientation de la Recherche Analytique Laitière
INRA : Institut National de la Recherche Agronomique
ITFF : Institut Technique Français des Fromages
LIAL MC : Laboratoire Interprofessionnel d'Analyses Laitières Massif Central
R & D : Recherche et développement

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