EVALUATION OF RIDABUTY (R-BIOPHARM) FOR THE ESTIMATION OF THE CONTAMINATION OF MILK WITH *CLOSTRIDIA* SPORES

Method tested

As with the reference method, the principle of numeration of the tested method is the calculation of the most probable number (MPN) from the number of wells revealed positive out of 5 inoculated at the dilution 0, and 5 at the dilution –1.
Ridabuty is used in this method for the detection of the positivity or negativity of inoculated wells. This instrument is based on the principle of nanorespirometry (with pressure and variable volume), and of the measurment of gas produced by bacterial development. It was set up by M. Bruno VERDIER of the CNRS (Patent n° 9001605) and AISSOR company (patent n° FR 0011555+PCT). It is distributed by R-Biopharm.

The method consists in the inoculation of microplates containing 96 wells, with 1 ml of test sample (milk or dilution –1) and 70µl of Bryant and Burkey broth modified by Bergère (medium described for the reference method). It is concentrated at 73,5 g/l in a HClN/3.5 solution. The dilution –1 is realised in UHT milk (it is therefore possible to do 8 analyses per plate). After thermisation by water jet spraying at 80°C under the plate for 13 minutes, an aluminium film is heat-sealed. The milk-medium mix in the plate is hand shaken and the plate is refrigerated and incubated in the press for 7 days at 37°C +/- 1.
The plates are read after centrifugation and piercing by passage in the Ridabuty instrument. It measures the gas flow (expressed in nanomoles/µl/hour) emitted by each well of the microplate.
These results are converted to positive or negative wells thanks to a threshold value (in gas flow) defined in the instrument software. The number of positive wells detected (for dilutions 0 and –1) permits then to quantify the result as a number of spores per litre of milk.

During this study, the evaluation of raw values has been realised with two threshold conditions :

1) a threshold fixed at –0.14 (corresponding to a real threshold flow value of –0.88 nanomoles/µl/hour) parametrised in the instrument for all the tests realised (identification in results tables : RESP –0.14).

2) a threshold fixed at –0.25 (corresponding to a real threshold flow value of –0.50 nanomoles/µl/hour). This threshold was chosen *a posteriori* in order to improve the test results. To choose this, all the raw test values were transformed into positive or negative values thanks to software provided by M. VERDIER (identification in results tables: RESP –0.25).

The tests

The evaluation tests were carried out in the microbiological laboratory at CECALAIT, from November 2004 to March 2005. They were realised with samples at ambient temperature without preliminary reheating. Specificity, linearity, repetability and accuracy of the method were evaluated.

The reference method for this study is the method described in the 2 following CNERMA (Centre National de Coordination des Etudes et Recherches sur la Nutrition et l'Alimentation) documents, published respectively in n° 451 (April 1986) and n° 469 (December 1987) of the Revue Laitière Française :

- Recommandations pour l'estimation de la contamination du lait en spores de *Clostridia* par la méthode de culture en milieu liquide.

- Recommandations pour établir les grilles de classements des laits en fonction de leur contamination en spores de *Clostridia.*

1- Evaluation of the specificity of the method :

The principle consists in the observation of the answer by the alternative method of "BUTYRIC" or "NON BUTYRIC" strains. Each strain is tested in sporulated form by the method to be evaluated and by the reference method.

15 "BUTYRIC" and 4 "NON BUTYRIC" strains were tested. Samples were provided from a suspension of about 50000 to 100000 spores / litre in raw milk supposedly free of spores.

The analyses were realised in duplicate by the reference method, with an intermediate reading at 5 days, and by the microrespirometry method with a final reading at 7 days of inoculation.
A check of the real level of spore contamination was realised on the samples to be tested after a thermisation of 15 minutes at 75°C by numeration on RCM agar 48 hours at 37°C in anaerobic conditions.

The specificity results for each strain are presented in table 1 in 2 different forms :

- Log value of the numeration obtained with the conversion table
- A quantitative interpretation determined by the results of spores / l as :

- : < 180 (2,26 in log)
+ : > 180 (2,26 in log) et < 2400 (3,38 in log)
++ : > 2400 (3,38 in log) and < 10000 (4,00 in log)
+++ : > 10000 (4,00 in log)

Table 1 : Results of specificity tests

« BUTYRIC » STRAINS

Genus species	N°	Strain reference	Log (spores/l)	Log REF	Log RESP -0,14	Log RESP -0,25	REF	RESP. -0,14	RESP. -0,25
C. tyrobutyricum	1	AQLC 3225	5,00	3,874	4,084	3,024	++	+++	+
	2	CNRZ608	4,75	3,081	3,244	2,846	+	+	+
	5	ADQ30L20	4,82	2,822	2,543	2,079	+	+	-
	9	CNRZ602	4,90	2,868	1,903	1,903	+	-	-
	10	CNRZ603	4,88	3,006	3,065	2,914	+	+	+
	11	CNRZ502	4,98	3,243	3,521	3,047	+	++	+
	12	CNRZ509	4,84	2,827	2,653	2,102	+	+	-
	13	ADQ39L26	4,83	3,329	1,903	1,903	+	-	-
	14	ADQ55L35	4,93	3,914	2,847	2,477	++	+	+
C. beijerenckii	3	CIP104308	5,69	3,387	4,380	3,889	++	+++	++
C. sporogenes	4	AQ94	4,81	3,447	3,496	3,345	++	++	+
	6	CL18	4,90	3,361	2,653	2,653	+	+	+
	7	35CL13	4,81	3,305	3,114	3,006	+	+	+
	15	2J021	4,60	3,247	3,211	3,110	+	+	+
	16	1G021	4,99	3,228	3,638	3,638	+	++	++

«NON BUTYRIC» STRAINS AND RAW MILK

Genus species	N°	Strain reference	Log (spores/l)	Log REF	Log RESP -0,14	Log RESP -0,25	REF	RESP. -0,14	RESP. -0,25
C. bifermentans	17	74483	4,90	2,976	2,923	2,923	+	+	+
C. perfringens	8	TQ049	4,61	3,136	1,903	1,903	+	-	-
Bacillus cereus	18	BC5	4,74	1,903	1,903	1,903	-	-	-
Bacillus polymyxa	19	PRF	4,94	2,884	3,964	3,964	+	++	++
Raw milk (8/11)	20(1)	Raw milk (1)	< 3,00	1,903	2,457	1,903	-	+	-
Raw milk(25/11)	20(2)	Raw milk (2)	< 3,00	1,903	2,884	2,102	-	+	-

Log (spores / l) : Log of the real value of the contamination (verification on solid medium)
Log REF : Log of the value obtained by the reference method
Log RESP –0,14 and –0,25 : Log of the value obtained by the method to be tested according to the two thresholds studied
REF, RESP –0,14 and –0,25 : qualitative interpretation

At a threshold RESP –0.14, the results of the Ridabuty method on the "BUTYRIC" strains are concordant with those of the reference method. However, the results obtained at the threshold RESP –0.25 are generally lower than those of the reference method.
Two strains at threshold –0.14 and 4 strains at threshold –0.25 of *C. tyrobutyricum* are negative with

the respirometry method and positive with the reference method.
For the 2 thresholds, the results with the Ridabuty method are negative for the "Non Butyric" strains, except for strains of *C. bifermentans* and *Bacillus polymysa* which are too positive with the reference method.
The concordance between the 2 methods for these "non butyric" strains is good.
Concerning the raw milk, the results are negative at the threshold –0.25 for the 2 methods (reference and respirometry) when they are positive at the threshold –0.14 with the respirometry method.

2- Linearity

The principle is to establish the relation (on milk samples prepared with a suspension of spores) between each method and the quantity of real spores in the sample (determined after thermisation 15 minutes at 75°C by numeration on RCM agar 48 hours at 37°C in anaerobic conditions).
3 strains of *Clostridium* (spore-forming) and concentrated silage were used. The concentrated silage was prepared by Cecalait with an herbage silage and conserved frozen. A suspension of about 300000 spores/l was prepared in raw milk supposedly spore-free. The suspension was then diluted in the raw milk (dilutions volume/volume) in order to cover an approximative range of 1000 to 300000 spores/l.

The analyses in duplicate for the dilutions 0 to –2 or –3 were carried out using the reference and the microrespirometry methods, with a final reading at 7 days of inoculation and an intermediary reading (5 days) for the reference method.
An evaluation of the real level of spore contamination by numeration on RCM agar 48 hours at 37°C in anaerobic conditions, after a thermisation 15 minutes at 75°C was realised on the sample presenting the higher level.
The samples constituted of 3 pure tested strains have a very low growth rate by the microrespirometry method at every level of contamination. Only the analysis of the samples realised with the concentrated silage by the microrespirometry method permits obtention of results corresponding to the real level of contamination and with the reference method.
The results obtained by the microrespirometry method (at the thresholds of –0.14 and –0.25) are proportional with the level of spores.

3- Evaluation of repeatability and accuracy

The milk was collected from the producers. These samples followed a normal routing for the payment of milk to quality and were sent to Cecalait by express carrier (arrival on the following morning before 12H). The samples were analysed at Cecalait simultaneously by the 2 methods. They came from 4 distinct regions concerning the payment classification.

The repeatability of the 2 methods was evaluated by the analysis in duplicate of approximately 100 milk samples from producers.

The comparison between the 2 methods was realised on about 280 milk samples from producers.

The accuracy was estimated by the residual standard deviation of regression (least squares regression) with :

- reference method (log spores/l) = explained variable Y
- Ridabuty (log spores/l) = explained variable X

Table 2 : Results of repeatability in log spores/l

	REFERENCE n = 102	**RESPIROMETRY n = 101***	
		Threshold at –0,14	**Threshold at –0,25**
log Sr	**0,23**	**0,33**	**0,30**

The results of repeatability obtained by the microrespirometry method are very close for the 2 tested thresholds. But, they are higher than the results obtained by the reference method. In the 2 cases, the observed standard deviation of repeatability is lower than the limit of 0.4 log defined in the CNIEL PROC CL-05-01/00 handbook.

* A value has been eliminated from the calculation for the respirometry method because of the deviation of 1.9 log at the threshold –0.14 between duplicates. This value has not been taken into account for the interpretation of accuracy.

Table 3 : Results of accuracy in log spores/l

	n = 281	
Positivity threshold	**RESP –0,14**	**RESP –0,25**
Mean deviation : RESP - REF	**0,16**	**-0,20**
Sd : Standard deviation of deviation	**0,57**	**0,51**
Sy,x : Residual standard deviation of regression	**0,54**	**0,48**

Figure 1 : Representation of the relation between the RIDABUTY method and the reference method at the threshold of –0,14

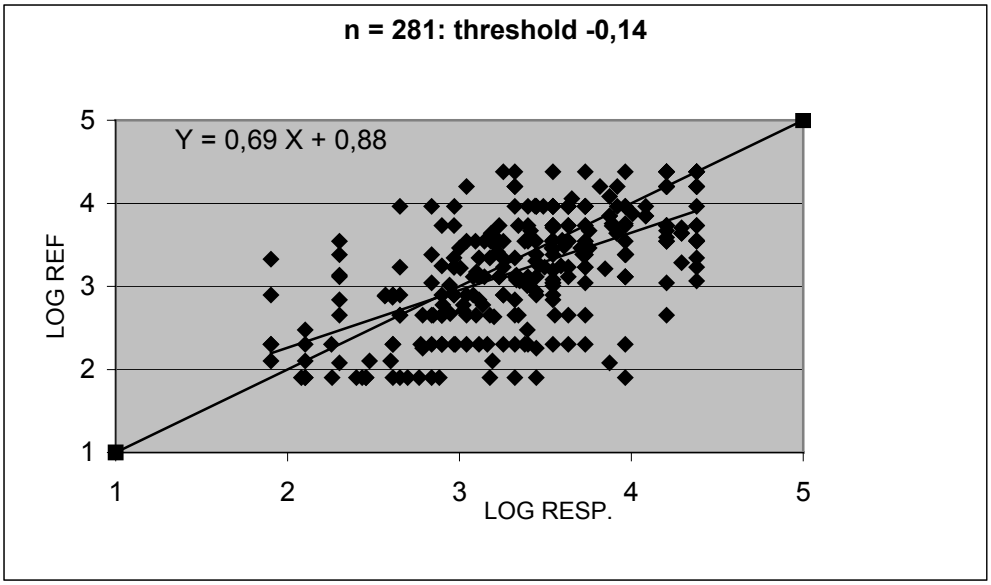
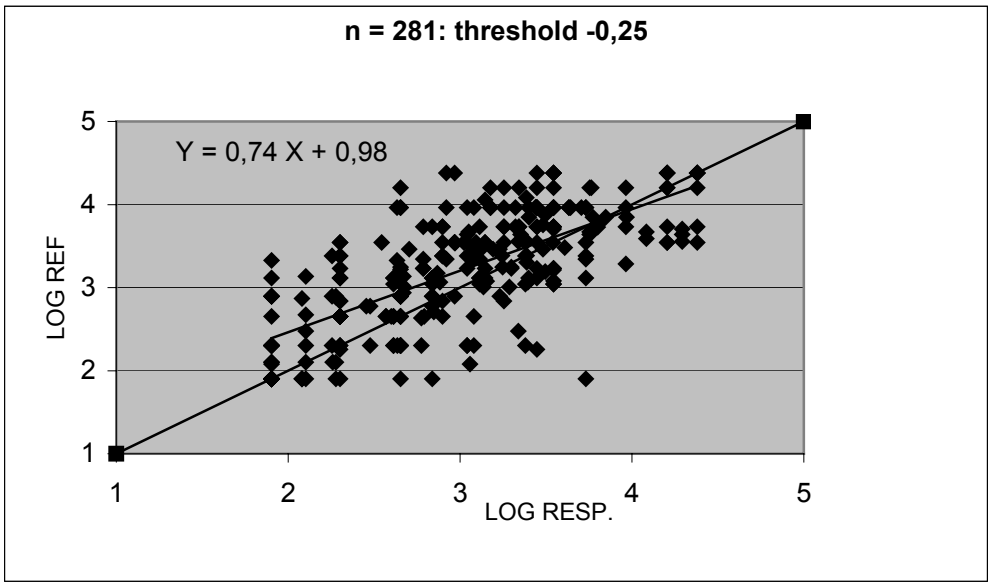


Figure 2 : Representation of the relation between the RIDABUTY method and the reference method at the threshold of –0,25



On the basis of the analysed samples, the configuration of the instrument with a threshold fixed at –0.25 permits to have a lower value of residual standard deviation of regression (and so precision of the estimation) of 0.48 log, instead of 0.54 log at a threshold of –0.14. There is also an inversion of the mean deviation from +0.16 to -0.20 log between the 2 methods.

Conclusion

The "positivity" threshold value chosen has a big importance on the performance of the method. Following the tests, with the threshold RESP –0.25, we obtain the best performance of linearity, repeatability and accuracy than with the threshold –0.14, which was initially programmed.
However, with this configuration, 4 pure strains of *C. tyrobutyricum* are not detected in the study of specificity, against 2 for the threshold RESP –0.14.Concerning the accuracy of the method (in relation to the reference method), the observed performances of this study are of the same order of magnitude as the values obtained at the time of a CNIEL test in 1995. This interlaboratory reproductibility test was carried out in 6 interprofessional laboratories with a total of 768 samples analysed in duplicate.
A residual standard deviation of regression (intra method CNERMA) of 0.51 log was obtained. For this evaluation, it was of 0.54 and 0.48 log, respectively, for the configuration RESP –0.14 and –0.25 (regression of the evaluated method in relation to the CNERMA method).

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