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Original article

Impedimetric method for selective enumeration of specific yoghurt bacteria with milk-based culture media

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Summary – The suitability of the impedimetric method for the selective enumeration of specific microorganisms, particularly yoghurt bacteria, *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp *bulgaricus*, was investigated in comparison with the standard plate count technique. A series of samples were analyzed by using new milk-based culture media (milk added with saccharose and acidified milk) for the impedance technique, whereas for the conventional counting method M17 and MRS culture media were applied. It was found that the milk-based culture media are suitable for the selective enumeration of specific yoghurt bacteria: the values detected by the impedimetric measurement (Bactometer instrument) and those resulting from the standard plate count showed a good correlation. The suggested milk-based culture media are shown to be suitable for the selective counting of yoghurt bacteria when applied in the impedimetric technique. Furthermore, since the bacterial counting may be determined within 12 h, the impedimetric method is also faster than the conventional method.

impedimetric method / selective enumeration / yoghurt / milk-based culture medium

Résumé – Méthode impédimétrique pour le dénombrement sélectif de la flore spécifique du yaourt en utilisant des milieux de culture à base de lait. *La validité de la méthode de l'impédance pour le dénombrement microbien de la flore spécifique du yaourt,* Streptococcus thermophilus *et* Lactobacillus delbrueckii *subsp* bulgaricus, *a été comparée avec la détermination des microorganismes réalisée par la méthode traditionnelle de numération en boîtes de Pétri. Plusieurs échantillons ont été analysés en appliquant les nouveaux milieux sélectifs de culture à base de lait (lait sucré et lait acidifié) pour ce qui concerne la méthode impédimétrique et en utilisant, parallèlement, les milieux de culture M17 et MRS pour la méthode de référence. On a vérifié que les milieux de culture à base de lait sont les plus appropriés pour la détermination de la population microbienne du yaourt par la mesure de l'impédance. Les donnés obtenues par la mesure de l'impédance et ceux de la méthode conventionnelle montrent une très bonne corrélation. Les milieux de culture à base de lait, en appliquant la méthode impédimétrique, peuvent assurer une détermination très simplifiée et rapide de la flore bactérienne du yaourt. Cette méthode est beaucoup plus rapide que la méthode de référence parce qu'on peut obtenir les données de la numération bactérienne dans les 12 h.*

méthode impédimétrique / dénombrement microbien / yaourt / milieu de culture à base de lait

INTRODUCTION

Bacterial enumeration carried out by the plate count technique (CFU), which was developed in the past century, has already been accepted as a reference procedure for the hygienic-sanitary and marketing evaluation of a product and, furthermore, is the only officially recognized method of analysis.

Nevertheless, the method has been shown not to be free from restrictions, mostly because of the long reaction times which sometimes do not comply with those of people working in a production sector. If that is the case, the end-product, in order to be marketed, should be manufactured according to the regulations and the laws in force.

The impedimetric technique has also been proposed in the past century, but only since the last 10 years it has been used as rapid counting method, alternatively with the standard plate count. The principle of the method, *ie* the measurement of the changes occurring in a substrate as evidence of a bacterial metabolism, has been demonstrated (Quesneau, 1983; Richardson, 1985): this is one of the points which mostly differs from the traditional plate count based upon the evaluation of the visible biomass (colonies).

The impedimetric technique is well known and usually applied in quality control laboratories of large industries, particularly for the total enumeration of microorganisms, estimation of contaminants and pathogens.

This technique allows to determine in a rapid, repeatable, accurate way and most of all, at lower costs, the shelf-life and bacteriological quality of raw materials for different food-products (Hardy *et al*, 1975; Williams and Wood, 1982; Zindulis, 1984; Easter and Gibson, 1985; Van Spreekens, 1986: Gibson, 1987; Banks *et al*, 1989) and particularly in meat (Martins and Selby, 1980; Firstenberg-Eden, 1983; Whittingham, 1989), milk (Gnan and Luedecke, 1982; O'Toole, 1983; Firstenberg-Eden and Tricarico, 1983; Nieuwenhof and Hoolfwerf, 1987), and other dairy products (Khayat *et al*, 1988; Story and White, 1988) as well as for those belonging to other production fields (pharmaceuticals, cosmetics, etc) (Kahn and Firstenberg-Eden, 1985; Edmondson, 1988).

Furthermore, the method is used for a rapid and accurate detection of bacteriophages in starter cultures and, generally speaking, in every fermentation process (Hose *et al*, 1983; Waes and Bossuyt, 1984).

Besides estimating the total bacterial count in a particular product such as yoghurt, is of great importance to determine the number of specific microorganisms, *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp *bulgaricus*, which must be present in high numbers and in a correct ratio.

In a previous study, Neviani *et al* (1991) obtained satisfactory results in the selective count of the two microorganisms specific for yoghurt by using the Malthus instrument and the modified culture media M17 and MRS.

The aim of the present study is to use milk-based culture media which are quite different from M17 and MRS. As well as being of easy preparation, they allow cost reduction in labour and materials and may be easily applied also in a production field.

MATERIALS AND METHODS

Cultures

Cultures used were: i) pure strains of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp *bulgaricus* belonging to the Centro Ricerche Yomo collection; ii) starters used for industrial manufacturing of yoghurt; and iii) yoghurt samples from factories.

Determination of lactic acid bacteria

Conventional method (FIL-IDF: 1986, 1988)

Bacterial content has been estimated by the count on plate of decimal dilutions in M17 and MRS culture media. MRS was acidified at pH 5.40 by addition of acetic acid.

The plates containing M17 culture medium, specifically developed for the enumeration of streptococci, were incubated at 37°C for 48 h, whereas MRS plates (pH 5.40), applied for the enumeration of lactobacilli, were incubated anaerobically at 37°C for 72 h.

Impedimetric method

The instrument Bactometer M 128 (Biomérieux, Rome, Italy) was used for measuring the detection time (DT) of the tested sample.

The DT is the time elapsing between the start of a test and the detection of an accelerating impedance signal by the instrument.

The DT is a function of numerous parameters, including the initial concentration of microorganisms, their lag phase, generation time and metabolic activity.

This relationship between DT and initial concentration of microorganisms allows the instrument to be used for the bacterial counting.

Selective enumeration by the impedance of Streptococcus thermophilus *and* Lactobacillus delbrueckii *subsp* bulgaricus *in mixed cultures*

Culture media

Skimmed milk powder (Oxoid, Unipath Ltd, Basingstoke, Hampshire, UK) reconstituted at 10% was modified as follows: i) by addition of 10, 15, 20% saccharose, and sterilizing in autoclave at 110°C for 20 min for streptococci test; ii) acidification at pH 5.00 by 2 N HCl, after sterilization in autoclave at 110°C for 10 min for lactobacilli test.

Determination of conductance curves

The impedimetric detection was carried out on samples at 24 and 72 h from their preparation.

Before being tested, the samples were diluted 1:10 in the culture medium to be applied for the evaluation and subsequently homogenized in Stomacher for 1 min.

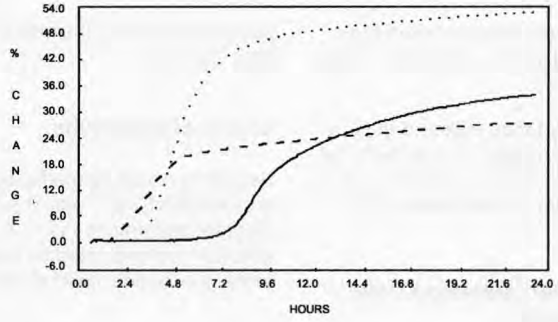

Fig 1. Conductance curves of a yoghurt culture in the following culture media: ..... milk; _____ milk + 15% saccharose; - - - acidified milk, pH 5.00.
Courbes de conductance d'une culture pour yaourt dans les milieux de culture suivants :..... lait ; _____ lait + 15% saccharose ; - - - lait acidifié pH 5,00.

Table I. Selectivity factor of milk + 15% saccharose for *Streptococcus salivarius* subsp *thermophilus* (inoculum 1:1000).
Facteur de sélectivité du lait +15% saccharose envers Streptococcus salivarius *subsp* thermophilus *(inoculum 1:1000).*

Yoghurt culture	*Initial CFU/ml (x 10^6)*	*Selectivity factor**
1		
St	0.890	20
Lb	0.194	1
2		
St	1.100	30
Lb	0.150	1
3		
St	0.830	40
Lb	0.145	1
4		
St	0.790	27
Lb	0.144	1
5		
St	0.510	48
Lb	0.163	1
6		
St	0.770	45
Lb	0.190	1

* Selectivity factor: N/N^0 relationship between the CFU/g at DT and initial CFU/g.

Table II. Selectivity factor of acidified milk (pH 5.00) for *Lactobacillus delbrueckii* subsp *bulgaricus (inoculum 1:1000).*
Facteur de sélectivité du lait acidifié (pH 5,00) envers Lactobacillus delbrueckii *subsp* bulgaricus *(inoculum 1:1000).*

Yoghurt culture	*Initial CFU/ml (x 10^6)*	*Selectivity factor**
1		
St	0.630	1
Lb	0.210	170
2		
St	5.450	1
Lb	0.150	500
3		
St	0.215	1
Lb	0.300	100
4		
St	0.855	1
Lb	0.230	200
5		
St	0.535	1
Lb	0.260	180
6		
St	0.300	1
Lb	0.380	100

* Selectivity factor: N/N^0 relationship between the CFU/g at DT and initial CFU/g.

Decimal dilutions were obtained from the initial dilution and placed into the incubator of the system (BPU).

The cultures in sweetened milk and those in acidified milk were incubated, respectively, at 42°C for the enumeration of streptococci, and at 47°C for the enumeration of lactobacilli (fig 1).

Relationship between detection time and microbial content

Detection times for all sample were correlated with the corresponding logarithm of the bacterial contents determined by the standard plate count. The linear regression of these data gives the calibration lines for the selective enumeration of both species.

Check of selectivity

Selectivity of culture media, both sweetened milk and acidified milk, after being inoculated with different percentages (1, 0.1, 0.01%) of yoghurt cultures was evaluated by using the plate count technique as described above.

Determination of a selectivity factor'

A selectivity factor N/N^o was established, where the two parameters N and N^o indicate, respectively, the CFU/g at the DT and the initial CFU/g.

Table III. Average values of bacterial enumeration with standard plate count and with impedimetric method.
Valeurs moyennes du dénombrement microbien en boîtes de Pétri et avec la méthode impédimétrique.

Yoghurt	*Sample number*	*Plate count (x 10^6/g)*		*Bactometer count (x 10^6/g)*	
		St	*Lb*	*St*	*Lb*
Natural whole milk	24	450	200	380	250
Whole milk apricot	20	180	200	300	260
Whole milk cherry	19	200	210	320	190
Whole milk citrus fruits	20	310	260	540	240
Whole milk ananas	18	240	225	210	195
Whole milk banana	22	280	230	360	190
Whole milk strawberry	26	260	230	420	240
Whole milk bilberry	22	300	250	360	230
Whole milk plum	14	260	220	350	260
Whole milk apple	9	330	310	310	300
Malt	18	240	300	260	290

The selectivity factor indicates the cellular growth of *Streptococcus thermophilus* or *Lactobacillus delbrueckii* subsp *bulgaricus*, inoculated 1:1000 in the corresponding culture media (milk added with saccharose or acidified milk) in the time period occurring between the incubation time and the DT.

RESULTS AND DISCUSSION

Milk-based culture medium added with saccharose for the selective enumeration of S thermophilus

It was observed that the addition of 15% saccharose to the milk allows to delay remarkably the development of *Lactobacillus delbrueckii* subsp *bulgaricus* without negatively affecting the growth of streptococci.

As shown in table I, in all six samples of yoghurt cultures the selectivity factor for lactobacilli and streptococci is substantially equal to 1. This is to show that their growth, as increasing number of cells, is practically insignificant. On the contrary, streptococci appear to develop regularly with a selectivity factor equal or higher than 20. Changes in conductance to determine the DT are therefore only due to the occurrence of streptococci.

Acidified milk-based culture medium for the selective enumeration of L delbrueckii *subsp* bulgaricus

It was found that by acidifying the milk and increasing the incubation temperature to 47°C, the streptococci growth was delayed without influencing the lactobacilli development. As shown in table II, in all six samples of yoghurt culture the selectivity factor for streptococci is substantially equal to 1. This suggests that their cellular growth is practically insignificant. Lactobacilli may, on the contrary, develop regularly and show selectivity factors equal or higher than 100. So changes in conductance to determine the DT are solely due to the occurrence of lactobacilli in the culture.

Table IV. Average values of bacterial enumeration with standard plate count and with impedimetric method.
Valeurs moyennes du dénombrement microbien en boîtes de Petri et avec la méthode impédimétrique.

Yoghurt	Sample number	*Plate count* ($x\ 10^6$/g)		Bactometer count ($x\ 10^6$/g)	
		St	*Lb*	*St*	*Lb*
Natural skim-milk	31	510	220	550	230
Skim-milk peach + passion fruit	13	550	240	590	250
Skim-milk strawberry + banana	12	500	200	660	200
Skim-milk ananas + grapefruit	13	600	250	540	220
Skim-milk orange + tangerine	13	600	220	500	200
Skim-milk plum	12	610	250	550	270
Skim-milk lemon	13	710	260	880	300
Skim-milk strawberry	11	650	280	640	260
Skim-milk wild fruits	10	690	260	610	250
Skim-milk natural corn	11	590	270	660	330

Comparative enumeration of lactic acid bacteria by using the standard plate method (Petri dishes) and impedimetric method

Ca 400 samples of yoghurt taken from storage-room at +4°C after 24 h of storage were analysed by the plate count and impedance to generate different calibration curves which correspond to the same number of products.

Because of the high number of samples as well as the heterogeneity of their composition (whole milk yoghurts, whole milk fruit yoghurt, skim-milk yoghurt) it is possible to verify the repeatability of the method and the reliability of the suggested culture media. In order the determine the repeatability of the method the standard ISO 5725 was used: the repeatability was 8% for streptococci and 20% for lactobacilli, largely below the 30% reported in Standard FIL 117A:1988.

From the tables III, IV and V it follows that a remarkable correspondence occurs between the values obtained by the traditional plate count technique and those calculated by the impedimetric method: this becomes more evident when the correlation coefficient (never below –0.98 for lactobacilli and –0.97 for streptococci) among the points of different calibration curves is evaluated (figs 2, 3). Furthermore, figures 2 and 3 show good correlation of values both for a low and high bacterial content.

CONCLUSIONS

From an evaluation of the results the two suggested culture media (sweetened milk and acidified milk) may be considered as suitable for the selective enumeration of specific yoghurt bacteria utilizing the impedance measurement detected by the Bactometer instrument.

Table V. Average values of bacterial enumeration with standard plate count and with impedimetric method.
Valeurs moyennes du dénombrement microbien en boîtes de Petri et avec la méthode impédimétrique.

Yoghurt with cream	Sample number	*Plate count (x 10^6/g)*		Bactometer count (x 10^6/g)	
		St	*Lb*	*St*	*Lb*
Type 1	17	380	7	390	9
Type 2	11	330	8	360	8
Type 3	21	390	7	350	7

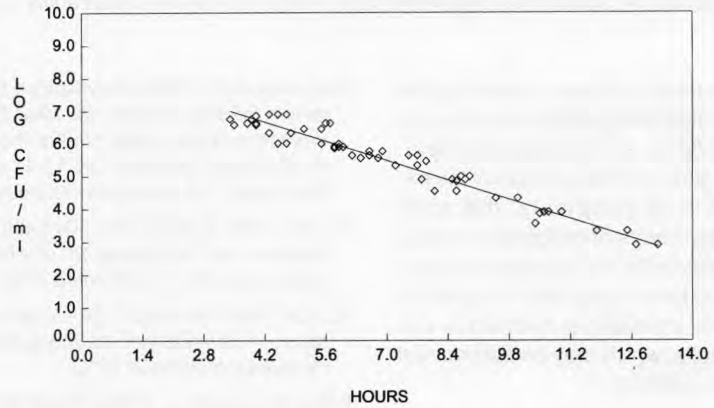

Fig 2. Regression line of *Streptococcus thermophilus* in whole milk yoghurt with strawberry. Log CFU/ml: -0.42*DT + 8.44. Correlation: -0.97.
Droite de régression de Streptococcus thermophilus *dans le yaourt entier à la fraise.*

The reliability of the proposed method is confirmed by the satisfactory, sometimes excellent, correspondence between the values detected by the Bactometer and those resulting from the plate count in factory both with high and low values of bacterial content.

Any under-evaluations or overestimations of the bacterial content are, in our opinion, due to the metabolic activity of the strains belonging to the specific yoghurt flora which is not so important for the plate count and, on the contrary, is very significant for the results detected by the Bactometer.

To conclude, the impedimetric method could represent an effective alternative to the traditional plate count technique since the data obtained by the impedimetric technique with their milk-based culture media are

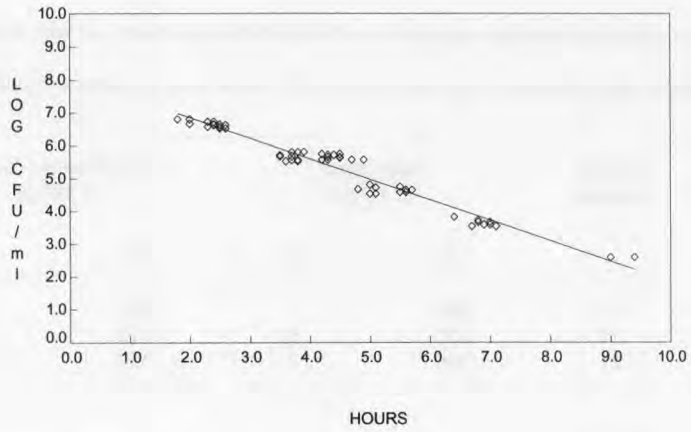

Fig 3. Regression line of *Lactobacillus delbrueckii* subsp *bulgaricus* in whole milk yoghurt with strawberry. Log CFU/ml: -0.62*DT + 8.05. Correlation: -0.98.
Droite de régression de Lactobacillus delbrueckii *subsp* bulgaricus *dans le yaourt entier à la fraise.*

largely comparable to those obtained by the conventional method. Furthermore, the culture media used for the selective enumeration provided good results because of the excellent factors of selectivity, the good correlation coefficients of calibration curves, the good repeatability, the easiness of preparation and the lower costs. Another point in favour of this innovative method is the rapidity of results, which may be determined in a short time, within 12 h.

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