A faster and more economical alternative to the standard plate count (SPC) method for microbiological analyses of raw milks

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According to the International Dairy Federation, the quality of raw milks shall be evaluated by the determination of "total" bacterial counts, or standard plate count (SPC); after serial dilution of the milk samples, the SPC is determined on Plate Count Agar (PCA) by the "pour plate" method**;** the viable bacteria are enumerated after aerobic incubation for 2 or 3 days, at 32 °C or 30°C respectively.

We analysed several raw milk samples and determined the CFU/ml, by the commonly used pour/ spread methods, and by the "drop method " which consisted of laying drops of non-diluted or diluted raw milks onto PCA plates (Miles and Misra method [1]). When enumerated by the drop method, the total counts were in average (based on

the analyses of 12 samples) 0.3-log lower then for the spread method. We believe that the "drop method", that presents the advantage of being faster and more economical, could be applied to raw milks, especially to studies where raw milks underwent certain treatments, accordingly where comparative enumerations between treated milks and controls needs to be performed.

Keywords raw milk; Standard Plate Count (SPC); Miles and Misra method

1. Introduction

Milk is a highly perishable foodstuff, and hence subjected to microbial contaminations; as milk is collected under different climates, by different handling practices, its microbiological quality is very variable, and the level of contaminations is reflected both in the number and types of microorganisms in the samples.

The evaluation of the microbial load of a sample, irrespective its nature, is performed by established methods: microscopy or plate counting (most currently the viable bacteria may be either determined by the pour plate, or by the spread plate methods) that enable direct analyses; indirect methods such as turbidometry, DNA-based techniques, immunological assays or more recently flow cytometry [2], fluorescence *in situ* hybridization (FISH, [3]) aim to be faster, more precise and culture-independant.

One method called Bactoscan, that is applied in Europe to analyse raw milks (although not yet approved for regulatory use in the U.S, [4]), enables to distinguish live and dead cells; it also discriminates individual cells from clusters; although very rapid, it seems however not to be the cheapest alternative to enumerate viable bacterial cells in milks.

The useful indicator that permits the monitoring of the sanitary conditions during the production, the collection and the handling of raw milks remains the "total" bacterial count or TBC, which is of interest for ensuring both quality and safety of raw milks [5].

The TBC is determined by the standard plate count (SPC), that estimates the aerobic and facultative anaerobic bacterial populations (by the pour plate method); the viable bacteria are enumerated on Plate

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Count Agar (PCA) after serial dilution of the raw milk, following aerobic incubation for 2 or 3 days, at 32 °C or 30°C respectively [6,7].

High SPC values of raw milks (> 10^5 CFU/ml) constitute evidence of hygiene deficiences; SPC values lower then 2.10^4 CFU/ml reflect good sanitary practices [5].

According to the IDF standards [8,9], other groups such as psychrotrophs or enterobacteria shall be enumerated similarly (by the pour plate method), considering specific incubation temperatures, or selective media.

The conventional plating methods (pour or spread), despite their shortenings, have the advantage of only counting living cells; the equipment necessary for performing viable plate counts is readily available to any microbiological laboratory, and is cheap comparatively to other methods.

In 1938, Miles and Misra proposed to "seed measured drops of culture on the surface of agar plates": several studies evaluated the precision of the drop plate method comparatively to other described methods; the authors considered whether pure cultures [10,11] or food products [12,13].

Considering the drop method, Miles and Misra (1938) already pointed its inconvenience; the size of the colonies (from pure cultures) was determining the colony counts that were readible. Some additional drawbacks were raised [14]: when considering the drop plate and spread plate methods, in both cases, low volumes (0.01 to 0.1 ml) are deposed on the agar plates; optical confusion can arise between food particles and small colonies; suspended food could also interfere by causing an inhibitory effect on microorganisms. Conversely, the pour plate, due to higher specimen volume (1ml) may be more satisfactory for samples with few organisms (less then 500 CFU/g) [14].

The pour plate method allows growth within the nutrient agar (facultative anaerobes), as well as on the surface; however, some relatively heat-sensitive microorganisms may be damaged by the melted agar. The spread plate method avoids this problem and enables the observation of various colony types more readily [14], as it allows also the selection of isolates.

Five methods of enumeration of microorganisms in food were evaluated [12] ; the surface drop method was the second less time-consuming (twice faster then the pour plate method).

Complementary to some extent, the pour and spread plate methods are time-consuming and costly (since a minimum of 9 plates is required to perform one analysis).

We evaluated here whether the drop method, slightly modified, would be applicable to raw milk samples for enumeration of total aerobes, anaerobes and psychrotrophs, considering its economy in materials and labour. The statistical analyses only concerned colonies that grew as strict aerobes, at 30ºC; hence the analyses were performed for the counts obtained by the spread and drop plate methods. The special interest consisted in finding a correction factor, that would enable to compare the two methods, and respond to the question whether the "total counts" determined by the drop plate method may be underestimated comparatively to those recorded by the spread plate method.

2. Materials and Methods

2.1 Analyses of raw milk samples

The raw milks (originating from lorry tanks) were serially diluted and analysed by the pour plate method (according to the IDF standard), by the spread method (50 µl of a dilution were spread onto a Plate Count Agar plate), by the drop method (20 µl of a dilution was laid onto a PCA plate and 3 dilutions were grouped inside one plate). Likewise for the aerobes, the level of strict anaerobes was determined by the spread and drop methods for three samples. The plates were incubated in an anaerobic jar with GENbox generators (bioMérieux) for 72h at 30ºC. The psychrotrophs were enumerated for three samples; the plates were incubated for 10 d at 7 ºC. For the pour and spread methods, the counts were recorded as recommended [15]. For the drop method, we considered only dilutions enabling to read distinct colonies, avoiding too low counts.

We further refer to the drop plate, pour plate and spread plate methods, as respectively DROP, POUR and SPREAD.

2.2 Statistical analyses

The averages of SPREAD and DROP (on log-scale, base = 10) were compared using paired t-test: in addition, POUR was compared to these two methods. In tables, the logarithmic averages are represented as original counts (CFU/ml) as well.

An additive correction factor was calculated for the results represented on log scale, and it was transformed to a multiplicative factor to be used when the data was expressed as CFU/ml. The potential precision of the correction factors was, due to a relatively small sample size, low. To take this in account, we calculated as well the one-sided confidence intervals for them. All analyses were performed using SAS, version 9.1.

3. Results and discussion

The log of the CFU/ml values resulting from the analyses of the raw milk samples, as obtained by the three methods, are given in Table 1.

Table 1 log CFU/ml, based on counts from three replicates (ND, not determined)
a) aerobes (SPREAD and DROP methods) and aerobes /facultative anaerobes (POUR method)

Sample	SPREAD	POUR	DROP
1	4,47	4,37	4,58
2	5,21	4,91	4,90
3	3,78	3,65	3,58
4	4,73	4,76	4,58
5	4,07	3,96	3,84
6	4,10	4,05	4,00
7	4,59	4,22	4,21
8	4,51	4,22	4,12
9	4,27	ND	3,86
10	3,76	ND	3,58
11	3,83	ND	3,50
12	4,17	ND	3,98

b) strict anaerobes

Sample	SPREAD	POUR	DROP
1	3,34	ND	3,26
2	5,08	ND	4,73
3	3,55	ND	3,42

c) psychrotrophs

Sample	SPREAD	POUR	DROP
1	2,97	2,76	3,00
2	4,96	4,50	4,70
3	3,10	2,71	2,82

When evaluated by the drop plate method (Table 1), the mesophilic aerobes were always lowest (with the exception of sample 1). On average, the difference was of 0.23-log between SPREAD and DROP; the difference between POUR and DROP was of 0.21, the counts being always lowest for DROP. This observation was also valid for the anaerobes that reach, irrespective the enumeration method, a slightly lower level then the aerobes: the differences was of 0.19-log between SPREAD and DROP.

Considering the psychrotrophs (Table 1), the counts recorded by the pour plate method were lowest; the differences between SPREAD and DROP, DROP and POUR being respectively of 0.17 and 0.19-log.

The comparison of the methods for the aerobic mesophiles are given below (Table 2).

Table 2 Statistical comparison of SPREAD and DROP averages of the aerobic mesophiles by paired t-test

Method		Average of CFU/ml		Standard deviation (log-scale)		Correction factor [d] for CFU/ml on	
		Counts	Log-scale (base 10)	Separate	Pooled	count	log-scale
SPREAD	n = 12	19534	4.2908	0.4330 [b]	0.4373	Estimated as average	
DROP		11503	4.0608	0.4416 [b]		1.698 [e]	0.2300
Difference	n = 12 (pairs)	41.12% [a]	0.2300 p = 0.0002	Standard error SE = 0.0427 [c]		Adjusted for noise [f]	
						1.996	0.3002
POUR	n = 8	18514	4.2675	0.4129 (SE = 0.1460)		-	

a DROP underestimates the total counts indicated as CFU/ml by this percentage as compared to SPREAD
b The standard deviations of the methods can not be regarded as different (p = 0.949)
c Calculated according to the paired design
d The values obtained by DROP should be multiplied with (count scale) or increased (on the log scale) by the given value to make them comparable to values obtained by SPREAD
e $10^{0.2300}$ -1
f These values take into account the sample size and the error variation. They are calculated as endpoints of one-sided 95% confidence interval

Based on the comparison of averages, as detailed in Table 2, DROP underestimated the total counts by 41%; hence its values have to be multiplied by 1.70 (i.e. increased by 70%) to make them comparable with SPREAD results. As the averages, on which this estimate is based, result from only 12 samples, one may shield against error inherent to the small number of considered samples (n = 12), by calculating the endpoint of confidence interval to be used as basis for the correction factor. With this additional caution taken the counts should be increased by 99.6%, i.e almost doubled; on log scale, the corresponding increase is 0.30.

We restricted the detailed analyses to the mesophilic strict aerobes; however, from data presented in Tables 1 and 2, the drop plate method may be of interest for enumerating other bacterial groups. Considering higher sample numbers, further analyses should be undertaken in order to determine whether similar correction factors could be determined and applied to the strict anaerobes and psychrotrophs.

As early as 1916, the difficulty "that food supply can be an issue, that colonies close to each other on the plate may merge, and that neighbor colonies may inhibit, or conversely stimulate the growth" was pointed [16]. If all methods aim to detect the "absolute" number of bacteria, no one is perfect. Plating, irrespective the methods are culture-dependant approaches, relying on differences in growth rates between different species.

The drop plate method, by lowering the costs of materials (reducing by 3 fold the number of necessary plates), for enumeration of the aerobes presents indeed some advantage; interestingly, it further lowers the costs of experiments when the anaerobes need to be included in studies, since less anaerobic generators are consumed.

It seemed to us that the drop method is not used for milk analyses; we believe, however, that it may be of interest for enumerating total aerobes, anaerobes, psychrotrophs, for studies where milks underwent

some treatment, where viable bacteria shall be enumerated from treated samples and controls, and especially where samples need to be analysed simultaneously, or with short delays.

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