Technical note: Enumeration of mesophilic aerobes in milk: Evaluation of standard official protocols and Petrifilm aerobic count plates

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ABSTRACT

Enumeration of mesophilic aerobes (MA) is the main quality and hygiene parameter for raw and pasteurized milk. High levels of these microorganisms indicate poor conditions in production, storage, and processing of milk, and also the presence of pathogens. Fifteen raw and 15 pasteurized milk samples were submitted for MA enumeration by a conventional plating method (using plate count agar) and Petrifilm Aerobic Count plates (3M, St. Paul, MN), followed by incubation according to 3 official protocols: IDF/ISO (incubation at 30°C for 72 h), American Public Health Association (32°C for 48 h), and Brazilian Ministry of Agriculture (36°C for 48 h). The results were compared by linear regression and ANOVA. Considering the results from conventional methodology, good correlation indices and absence of significant differences between mean counts were observed, independent of type of milk sample (raw or pasteurized) and incubation conditions (IDF/ISO, American Public Health Association, or Ministry of Agriculture). Considering the results from Petrifilm Aerobic Count plates, good correlation indices and absence of significant differences were only observed for raw milk samples. The microbiota of pasteurized milk interfered negatively with the performance of Petrifilm Aerobic Count plates, probably because of the presence of microorganisms that poorly reduce the dye indicator of this system.

Key words: milk, mesophilic aerobe, enumeration method, Petrifilm

The quality of milk depends on efficient control during various stages of production, storage, transport, processing, and commercialization. In each of these stages, specific conditions must be properly and constantly monitored, because they can jeopardize the final quality of milk and dairy products. To guarantee adequate monitoring, governmental agencies are responsible for

determining parameters and rules that must be followed during all steps of production. These organizations are also responsible for systematic verification of whether these parameters and rules are being met.

In Brazil this regulatory role is conducted by the Ministry of Agriculture [Ministério da Agricultura, Pecuária e Abastecimento (**MAPA**); Brasil, 2008a] and by the Ministry of Health through the National Agency of Sanitary Vigilance [Ministério da Saúde, Agência Nacional de Vigilância Sanitária (**ANVISA**); Brasil, 2008b]. An example of these regulations was the Instrução Normativa 51, from MAPA, which was published in 2002 establishing several quality parameters of animal health and quality of raw and pasteurized milk (Brasil, 2002).

The mesophilic aerobes (**MA**) are indicator microorganisms often used to verify the microbiological quality of milk and dairy products, because they provide valuable information about sanitary and hygienic conditions of milking, storage, and processing, and also suggest the presence of pathogenic microorganisms (ICMSF, 1988). Mesophilic aerobes are able to grow between 20 and 45°C, with an optimum growth temperature of 32°C, and include the majority of spoilage and pathogenic microorganisms associated with milk and dairy products (Jay et al., 2005).

Enumeration of MA is conventionally done by pour plating diluted samples in a nonselective culture medium, usually plate count agar, followed by incubation in aerobic conditions at mesophilic temperatures for a specific period of time (Robinson, 2002). Considering these requirements, several combinations of these conditions are described in standard protocols for MA enumeration in foods, including dairy products. The International Dairy Federation (**IDF**) recommends the incubation of plates at 30°C for 72 h (ISO, 2003); the American Public Health Association (**APHA**) sets the incubation at 32°C for 48 h (Wehr and Frank, 2004); and MAPA recommends incubation at 36°C for 48 h (Brasil, 2003).

Although it is the official benchmark, traditional microbiological analysis has limited application because it cannot provide instantaneous results for immediate

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Table 1. Mean counts ($\pm$ SD) of mesophilic aerobes ($\log_{10}$ cfu/mL) in raw and pasteurized milk samples obtained by conventional and Petrifilm AC plating¹ and incubated at 3 distinct conditions

Category	Incubation conditions		
	30°C, 72 h	32°C, 48 h	36°C, 48 h
	ISO (2003)	APHA (2004)	Brasil (2003)
Raw milk			
Conventional plating	7.63 $\pm$ 0.29 ^{a,A}	7.53 $\pm$ 0.35 ^{a,A}	7.38 $\pm$ 0.45 ^{a,A}
Petrifilm AC plating	7.69 $\pm$ 0.27 ^{a,A}	7.48 $\pm$ 0.35 ^{a,A}	7.33 $\pm$ 0.44 ^{a,A}
Pasteurized milk			
Conventional plating	4.14 $\pm$ 0.10 ^{a,A}	3.99 $\pm$ 0.14 ^{a,A}	3.89 $\pm$ 0.17 ^{a,A}
Petrifilm AC plating	4.05 $\pm$ 0.10 ^{a,A}	3.51 $\pm$ 0.43 ^{b,B}	2.33 $\pm$ 0.48 ^{c,B}

^{a-c}Means within a row with different superscript lowercase letters differ ($P < 0.05$).

^{A,B}Means within a column in each category with different superscript uppercase letters differ ($P < 0.05$).

¹Petrifilm Aerobic Count plate (3M, St. Paul, MN).

or rapid corrective actions (Fung, 2002). In addition, traditional microbiological methods demand preparation of culture media and glassware, requiring valuable work time and material from laboratories (Park et al., 2001; Sant'Ana et al., 2002). Considering these limitations, several alternative microbiological methodologies were developed in the 1970s, known as rapid methods (Fung, 2002). The Petrifilm system (3M Microbiology, St. Paul, MN) is one of these rapid methods and is widely used by food industries for monitoring microbiological contamination of food processing and final products (Blackburn et al., 1996). Several microorganism groups can be enumerated by distinct presentations of the system, and the Petrifilm Aerobic Count (AC) plates are commonly used for enumeration of MA.

Considering the need for reliable and adequate enumeration of MA in milk to provide proper quality control during production and processing, we compared the protocols of IDF/ISO, APHA, and MAPA for MA enumeration in raw and pasteurized milk samples, using the conventional plating methodology and Petrifilm AC plates.

Milk samples ($n = 15$ of raw milk and $n = 15$ of pasteurized milk) were aseptically collected in a dairy industry in Viçosa (Minas Gerais, Brazil). All samples were kept under refrigeration until analysis, when they were submitted to 10-fold serial dilution using 0.85% NaCl for microbiological procedures.

All diluted samples were submitted to MA enumeration by conventional plating and using Petrifilm AC plates, followed by incubation in different combinations of temperatures and periods of incubation, according to protocols described by IDF/ISO (ISO, 2003), APHA (Wehr and Frank, 2004), and MAPA (Brasil, 2003). Two dilutions from each sample were selected and pour plated with plate count agar (Becton Dickinson, Franklin Lakes, NJ) in duplicate. The same dilutions

were plated in Petrifilm AC without replicates. The described plating procedures were conducted in triplicate for each sample, considering the distinct conditions of incubation described as follows.

Each triplicate plate (conventional and Petrifilm AC) was incubated under distinct conditions, according to IDF/ISO: 30°C for 72 h (ISO, 2003); APHA: 32°C for 48 h (Wehr and Frank, 2004); and MAPA: 36°C for 48 h (Brasil, 2003). After incubation, the colonies were enumerated and the final results expressed in colony-forming units per milliliter.

The obtained counts were converted to $\log_{10}$ and the mean values were compared by ANOVA to identify significant differences between the protocols and enumeration systems (conventional plating and Petrifilm; $P < 0.05$). In addition, the data were compared by correlation considering the same variables. All statistical analyses were processed using Statistica 7.0 software (StatSoft Inc., Tulsa, OK).

The mean values of MA obtained from raw and pasteurized milk samples by conventional plating and Petrifilm AC in each evaluated incubation condition are presented in Table 1. Considering only the results obtained by the conventional procedure, the obtained data indicated equivalency between the incubation conditions for all types of samples, because no significant differences were observed ($P > 0.05$). Considering Petrifilm AC, no differences between the mean counts obtained under each incubation condition were observed for raw milk samples ($P > 0.05$). However, for pasteurized milk samples, the mean values obtained at 30°C for 72 h were significantly higher compared with the mean value at 32°C for 48 h, which was also significantly higher than the mean value obtained at 36°C for 48 h ($P < 0.05$).

Considering the comparison between the mean counts obtained in each system and incubation condition, sig-

Table 2. Statistical correlation parameters between mesophilic aerobes counts of raw and pasteurized milk obtained by conventional plating or Petrifilm system incubated at 3 distinct conditions

Comparisons (x:y)	Statistical correlation parameters ¹						
	n	r	R ²	P	a	b	mv
Conventional plating							
30°C, 72 h: 32°C, 48 h	29	0.99	0.98	0.000	1.02	-0.22	0.03
30°C, 72 h: 36°C, 48 h	28	0.99	0.98	0.000	0.99	-0.24	0.07
32°C, 48 h: 36°C, 48 h	29	0.99	0.98	0.000	0.98	-0.01	0.04
Petrifilm AC ² plating							
30°C, 72 h: 32°C, 48 h	30	0.99	0.97	0.000	1.09	-0.89	0.14
30°C, 72 h: 36°C, 48 h	30	0.99	0.97	0.000	1.37	-3.18	0.85
32°C, 48 h: 36°C, 48 h	30	0.97	0.94	0.000	1.22	-1.87	0.50

¹n = number of repetitions; r = correlation index; R² = determination coefficient; P = level of significance; a = slope; b = intercept; mv = mean variance.

²Petrifilm Aerobic Count plate (3M, St. Paul, MN).

nificant differences were only observed for pasteurized milk at 32°C for 48 h and 36°C for 48 h ($P < 0.05$; Table 1). The mean counts of MA obtained from raw milk samples at all incubation conditions, and pasteurized milk at 30°C for 72 h did not present significant differences ($P > 0.05$).

These results indicate that at higher incubation temperatures for 48 h, a portion of pasteurized milk microbiota was not able to form visible colonies in Petrifilm AC plates, resulting in lower counts compared with the conventional plating procedure and incubation at 30°C for 72 h (Table 1). The probable cause for these differences is the presence of naturally occurring microbiota in pasteurized milk with low capability of reducing 2,3,5-triphenyltetrazolium chlorine (TTC; Beloti et al., 2002), the dye indicator used in Petrifilm AC plates to identify the formed colonies. Thermotolerant microorganisms are typically present in pasteurized milk and they are known to be poor TTC reducers (Beloti et al., 1999).

The equivalency of incubation conditions by conventional plating procedure was confirmed by the data presented in Table 2. In all possible comparisons between the obtained results in each incubation condition, it was possible to verify excellent correlation indices. Considering the results obtained only by Petrifilm AC plates, interferences can be observed when the results from distinct incubation conditions are compared, mainly by inadequate values of slope (a) and intercept (b) (Table 2). When these data are compared considering only the plating system, the counts obtained from Petrifilm AC plates incubated at 30°C for 72 h presented excellent correlation indices with data obtained in distinct incubation conditions by conventional plating (Figure 1). However, as the incubation temperature increased, the slope (a) and intercept (b) varied and indicated some deficiencies on the performance of Petrifilm AC (Figure

1). These statistical parameters are useful to evaluate the correlation between the methodologies.

These data confirm the significance of pasteurized milk microbiota on the performance of Petrifilm AC. Beloti et al. (1999) reported that the majority of nonreducing TTC microorganisms are thermotolerant, predominantly gram-positive bacteria (95.7%), typically found in pasteurized milk. Nonetheless, several species of naturally occurring microorganisms in milk and dairy products are known for their low capability of reducing TTC (Swanson et al., 1992). These microorganisms are incapable of forming visible colonies in Petrifilm AC plates, and are not consequently enumerated. *Streptococcus viridans* is one of these microorganisms, and it is not able to produce characteristic red colonies in Petrifilm AC plates when incubated at 32°C for 48 h, requiring 5 d for this (Dawkins et al., 2005). Other species from *Streptococcus* genera were described previously as poor or nonreducing TTC, such as *Streptococcus cremoris* (Turner et al., 1963) and *Streptococcus thermophilus* (Dawkins et al., 2005; Nero et al., 2006; Ortolani et al., 2007).

The obtained results suggest that 72 h of incubation is the required incubation period for Petrifilm AC plates for adequate development of colonies from microorganisms with low capability of reducing TTC from pasteurized milk. Considering this, the absence of difference between the mean counts obtained from Petrifilm AC plates and the conventional procedure (Table 1) and the better correlation indices (Figure 1) are adequately explained.

Despite the observed limitations related to Petrifilm AC performance for MA enumeration in pasteurized milk produced in Brazil (Beloti et al., 2002; present study), the convenience of this system for microbiological analysis in raw milk and other foods has been reported in previous studies (Park et al., 2001; Ellis and

Figure 1. Dispersion of mesophilic aerobes counts in raw and pasteurized milk obtained by conventional (x-axis) and Petrifilm Aerobic Count (y-axis; Petrifilm, 3M, St. Paul, MN) plating and incubated at 3 distinct conditions [30°C for 72 h (ISO, 2003); 32°C for 48 h (American Public Health Association; Wehr and Frank, 2004); and 36°C for 48 h (Brasil, 2003)]. In each graphic: $y = ax + b$ [correlation formula, indicating slope (a) and intercept (b)]; correlation index (r), determination coefficient (r^2); number of repetitions (n); mean variance (mv); and level of significance (P).

Meldrum, 2002; Tavolaro et al., 2005). The microbiota of the tested food is a determining factor for the performance of Petrifilm, but distinct incubation conditions can improve the efficiency of this system.

The obtained results indicate that the 3 tested incubation conditions can be similarly used for MA enumeration in raw and pasteurized milk by conventional plating procedures. As an alternative, Petrifilm AC plates can be used for MA enumeration in raw milk samples in any of the test incubation conditions. For pasteurized milk, significant interference of the autochthonous microbiota on the performance of Petrifilm AC was verified, which was partially corrected when the plates were incubated at 30°C for 7 h. This condition apparently allowed for the adequate development of visible colonies from poor or nonreducing TTC microorganisms.

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