Journal of Dairy Research (2007) **74** 387–391. © *Proprietors of Journal of Dairy Research 2007*
doi:10.1017/S002202990700266X *First published online 9 July 2007 Printed in the United Kingdom*

Screening and enumeration of lactic acid bacteria in milk using three different culture media in Petrifilm™ Aerobic Count plates and conventional pour plate methodology

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Received 12 December 2006 and accepted for publication 27 March 2007

This study aimed to compare Petrifilm™ Aerobic Count (AC) plates and the conventional pour plate methodology using de Mann-Rogosa-Sharpe (MRS), Kang-Fung (KF) and Kang-Fung-Sol (KFS) culture media for screening and enumeration of lactic acid bacteria (LAB) in milk. Suspensions of 10 LAB species in reconstituted powder skim milk and 30 raw milk samples, without experimental inoculation, were tested. For selective enumeration, all samples were previously diluted in MRS, KF and KFS broths and then plated in Petrifilm™ AC and conventional pour plate methodology, using the same culture media with added agar. All plates were incubated at 37 °C for 48 h in anaerobic conditions. Differences in the counts were observed only for raw milk samples using KFS in conventional methodology, when compared with the counts obtained from MRS and KF ($P \leqslant 0{\cdot}05$). The results showed excellent correlation indexes between both methodologies using the three culture media for LAB suspensions (r=0·97 for MRS, KF and KFS). For raw milk samples, the correlation indexes were excellent (r=0·97, for MRS) and good (r=0·84 for KF, and r=0·82 for KFS), showing some interference in Petrifilm™ AC when supplements were added, especially lactic acid. These results indicate the possibility of using Petrifilm™ AC plates for enumeration of LAB in milk, even with the use of selective supplements.

Keywords: Lactic acid bacteria, Petrifilm™, de Mann-Rogosa-Sharpe, Kang-Fung, Kang-Fung-Sol.

Lactic acid bacteria (LAB) are microorganisms related to various habitats and are known as probiotics in food and pharmaceutical industries (Holzapfel et al. 2002). They are used as starter cultures in meat and dairy products (Salminen et al. 2004) and have important antagonistic activity against pathogens in food (de Martinis et al. 2002). These applications show the significance of LAB contribution to microbial safety in food, in addition to organoleptic, technological and nutritional advantages that they offer (Leroy & de Vuyst, 2004).

Screening and enumeration of LAB in food products is usually done using de Mann-Rogosa-Sharpe (MRS) agar under microaerophilic or anaerobic conditions (Wher & Frank, 2004), but non-LAB microorganisms can growth in this culture media due to its various nutrients and the absence of selective agents (Kang & Fung, 1998). One way to provide selectivity to MRS is the addition of supplements such as phenylethanol and lactic acid, which are used in Kang-Fung (KF) and Kang-Fung-Sol (KFS) culture media to inhibit the growth of Gram-negative bacteria and Gram-positive pathogens, such as *Listeria monocytogenes* and *Staphylococcus aureus* (Kim et al. 2001).

Petrifilm™ plates (3M Microbiology, St. Paul, MN) are widely used in the food industry for monitoring the microbiological quality of food products (Byrne & Bishop, 1991; Beuchat et al. 1998; Ferrati et al. 2005; Tavolaro et al. 2005). These plates are commercialized in a double layer format that allows their ready use, simply by dilution and direct inoculation of the sample to be tested. Petrifilm™ Aerobic Count (AC) plates are conventionally used for mesophilic aerobic enumeration, but also can be used for LAB enumeration, particularly in fermented products (Champagne et al. 1994; McGregor et al. 1995; Nero et al. 2006). For this purpose, the manufacturers of Petrifilm™ plates recommend dilutions of samples in

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https://doi.org/10.1017/S002202990700266X Published online by Cambridge University Press

Table 1. Lactic acid bacteria strains used in the study

Strains	Reference†
Enterococcus faecalis	ATCC 19843
Enterococcus faecium	ITAL
Lactobacillus delbrueckii subsp. *bulgaricus*	ATCC 11842
Lactobacillus delbrueckii subsp. *delbrueckii*	ATCC 9649
Lactobacillus paracasei subsp. *paracasei*	ATCC 10746
Lactococcus lactis subsp. *cremoris*	NCDO 1428
Lactococcus lactis subsp. *lactis*	ATCC 7962
Leuconostoc lactis	ATCC 19256
Pediococcus pentosaceus	LMI-BIOAGRO
Streptococcus thermophilus	NCDO 1968

† ATCC, American Type Culture Collection, Manassas, VA, USA. ITAL, Institute of Food Technology, Campinas, SP, Brazil. NCDO, National Collection of Dairy Organisms (currently, National Collection of Food Bacteria, Aberdeen, Scotland). LMI-BIOAGRO, Industrial Microbiology Laboratory (LMI, Bioagro), Universidade Federal de Viçosa, Viçosa, MG, Brazil.

conventional methods and the use of MRS broth in the final dilution step, followed by incubation at microaerophilic or anaerobic conditions. However, no information is available on possible interference of selective agents added to Petrifilm™ AC plates for proper LAB screening and enumeration in foods.

This study aimed to evaluate Petrifilm™ AC plates and conventional pour plate methodology for screening and enumeration of 10 different species of LAB inoculated in reconstituted powder skim milk, using three different culture media: MRS, KF and KFS. To verify the reproducibility of Petrifilm™ AC performance, raw milk samples with no experimental LAB inoculation were also tested.

Material and Methods

Strains and inocula preparation

The microbial strains used in this study were reference LAB cultures (Table 1), maintained in MRS agar (Oxoid, Basingstoke, U.K.) slants under refrigeration. For use, each strain was plated on MRS agar and incubated at 30 °C for 24 h in microaerophilic conditions. One isolated colony was transferred to tubes containing MRS broth (Oxoid) and incubated at 30 °C for 24 h. Cultures in MRS broth were submitted to decimal dilutions in NaCl solution (8·5 g/l), and the dilution with turbidity equivalent to McFarland scale 1 was selected. This dilution was then submitted again to decimal dilutions in NaCl solution to obtain a culture with approximately 10^5 cells/ml.

Preparation of experimental LAB suspensions

Powdered skim milk (Molico, Nestlé, São Paulo, Brazil) was reconstituted in recently sterilized distilled water (100 g/l), distributed in sterile flasks (100 ml per flask), and 1 ml of each LAB culture to be tested was added. After homogenization, the flasks were kept under refrigeration for 24 h and then submitted to microbiological analysis. Immediately before testing, the samples were thoroughly homogenized.

Counting of LAB in the suspensions

The suspensions of LAB in reconstituted milk were submitted to serial decimal dilutions in NaCl solution (8·5 g/l) following by an additional dilution in MRS, KF and KFS broths. The dilutions were then pour plated in MRS, KF and KFS agars, respectively, and in Petrifilm™ AC plates, following the recommendations of the manufacturer. The plates were placed in anaerobic jars with anaerobic generators (Anaeropac, Probac do Brasil, São Paulo, S.P., Brazil) and were incubated at 37 °C for 48 h. Plates containing 25–250 colonies were selected, and the colonies were enumerated. The number of colony-forming units per milliliter milk sample (CFU/ml) was calculated.

The culture media used in this study were prepared according to Kim et al. (2001). MRS medium was obtained from Oxoid, KF was prepared by adding phenylethanol (1·75 ml/l) (VETEC, São Paulo, S.P., Brazil) into a MRS medium and KFS was prepared by adding phenylethanol (1·75 ml/l) and lactic acid (2 ml/l) (VETEC) to MRS medium, to reduce the pH to 5·5. For conventional plating, agar (Oxoid) was added to each medium prior to autoclaving (15 g/l). Phenylethanol and lactic acid were added after autoclaving and pH was ascertained after sterilizing and cooling at room temperature.

Counting of naturally occurring LAB

Raw milk samples ($n=30$), obtained from dairy industries located in the region of Viçosa, M.G., Brazil, were thoroughly homogenized, submitted to serial dilutions in NaCl solution (8·5 g/l) and then MRS, KF and KFS broths, and pour plated in MRS, KF and KFS agars, respectively, and Petrifilm™ AC plates, using the same incubation and counting procedures described for the experimental suspensions.

Statistical analysis

The experiments with the suspensions of LAB in reconstituted milk were repeated at least three times. The average colony counts, expressed as log CFU/ml, were subjected to regression analysis. Also, the results were compared by Tukey's HSD test, considering $P \leqslant 0{\cdot}05$ as significance differences between the two evaluated methods and three culture media. Both tests were done using Statistica 6.0 software (Stat Soft, Inc., Tulsa, OK).

Results and Discussion

Results of mean counts of each LAB species and raw milk samples plated in conventional methodology and

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Table 2. Means counts of Lactic Acid Bacteria and raw milk samples using MRS, KF and KFS culture media in conventional plating and Petrifilm™ AC plates

Values are mean counts (CFU/ml) ± SD for *n* = number of repetitions

Testing material	Repetitions *n*	Mean counts ± standard deviation (log_{10} CFU/ml)					
		MRS agar	KF agar	KFS agar	Petrifilm™ AC with MRS broth	Petrifilm™ AC with KF broth	Petrifilm™ AC with KFS broth
Suspensions	33	4·28 ± 0·39^{a}	4·28 ± 0·36^{a}	4·27 ± 0·36^{a}	4·29 ± 0·36^{a}	4·28 ± 0·37^{a}	4·26 ± 0·37^{a}
Enterococcus faecalis	3	4·44 ± 0·12^{a}	4·47 ± 0·08^{a}	4·46 ± 0·12^{a}	4·47 ± 0·13^{a}	4·59 ± 0·15^{a}	4·44 ± 0·06^{a}
Enterococcus faecium	5	3·98 ± 0·11^{a}	4·04 ± 0·11^{a}	4·05 ± 0·09^{a}	3·98 ± 0·09^{a}	4·05 ± 0·10^{a}	4·00 ± 0·16^{a}
Lac. delbrueckii subsp. *bulgaricus*	3	4·28 ± 0·57^{a}	4·15 ± 0·41^{a}	4·11 ± 0·41^{a}	4·17 ± 0·32^{a}	4·18 ± 0·41^{a}	4·20 ± 0·43^{a}
Lac. delbrueckii subsp. *delbrueckii*	3	3·95 ± 0·34^{a}	3·94 ± 0·36^{a}	3·93 ± 0·32^{a}	3·97 ± 0·23^{a}	3·92 ± 0·42^{a}	3·92 ± 0·31^{a}
Lac. paracasei subsp. *paracasei*	3	4·40 ± 0·08^{a}	4·41 ± 0·07^{a}	4·44 ± 0·09^{a}	4·39 ± 0·06^{a}	4·35 ± 0·06^{a}	4·37 ± 0·10^{a}
Lactococcus lactis subsp. *cremoris*	4	4·50 ± 0·26^{a}	4·44 ± 0·25^{a}	4·39 ± 0·24^{a}	4·53 ± 0·29^{a}	4·41 ± 0·35^{a}	4·46 ± 0·29^{a}
Lactococcus lactis subsp. *lactis*	3	4·11 ± 0·04^{a}	4·12 ± 0·03^{a}	4·15 ± 0·02^{a}	4·11 ± 0·04^{a}	4·15 ± 0·07^{a}	4·09 ± 0·50^{a}
Leuconostoc lactis	3	4·76 ± 0·62^{a}	4·78 ± 0·59^{a}	4·73 ± 0·65^{a}	4·69 ± 0·64^{a}	4·67 ± 0·62^{a}	4·63 ± 0·63^{a}
Pediococcus pentosaceus	3	4·11 ± 0·03^{a}	4·08 ± 0·08^{a}	4·12 ± 0·14^{a}	4·21 ± 0·14^{a}	4·17 ± 0·12^{a}	4·12 ± 0·07^{a}
Streptococcus thermophilus	3	4·42 ± 0·66^{a}	4·44 ± 0·60^{a}	4·43 ± 0·63^{a}	4·45 ± 0·66^{a}	4·46 ± 0·61^{a}	4·47 ± 0·67^{a}
Raw milk samples	30	5·47 ± 1·02^{a}	5·15 ± 0·92^{a}	4·07 ± 1·03^{b}	5·35 ± 0·92^{a}	5·25 ± 1·01^{a}	4·77 ± 0·73^{a,b}

^{a,b}Values in the same row with common superscript letters are not significantly different (Tukey's HSD test, $P \leqslant 0·05$)

Petrifilm™ AC with MRS, KF and KFS media are shown in Table 2. For LAB suspensions the differences between results of both methodologies were not significant, at a level of significance of 0·05, regardless of the species tested. However, for raw milk samples the mean count obtained in KF and KFS media were lower compared with the results in MRS, but only in the conventional methodology using KFS was this difference significant ($P \leqslant 0·05$). The lower counts in raw milk samples using KF and KFS media occurred due to the addition of the selective substances phenylethanol and lactic acid. According to Kim et al. (2001), KFS medium has a greater selectivity potential when compared with MRS and KF due to the addition of an organic acid.

When the mean counts of LAB from each culture media were compared between both methodologies, no significant differences were observed ($P \geqslant 0·05$), indicating equivalency as previously described (Champagne et al. 1994; McGregor et al. 1995; Nero et al. 2006). But, the mean counts of microorganisms in raw milk obtained in KF and KFS media using Petrifilm™ AC plates tended to be higher than those obtained by the conventional methodology, suggesting a smaller interference of the added supplements in the first method. Ferrati et al. (2005) found a similar interference in Petrifilm™ AC plates in an evaluation of this method to enumerate microorganisms from acid juices.

Figure 1 shows the dispersion of the counts of LAB species and raw milk samples obtained in MRS, KF and KFS media using Petrifilm™ AC and conventional pour plate methodology. These results indicate an excellent correlation (r=0·97) between results obtained in both methodologies using MRS media for LAB strains and raw milk samples. For KF and KFS media, the results indicate excellent correlation between methodologies when LAB strains are tested (r=0·97 for both cases), but lower correlation indexes for enumeration of microorganisms from raw milk samples (r=0·84 for KF, r=0·82 for KFS). For these culture media, the correlation indexes between Petrifilm™ AC and the conventional methodology can be classified as good. All obtained correlations were highly significant ($P=0·00$ in all cases, Fig. 1).

The convenience of using Petrifilm™ AC plates associated to MRS broth for LAB enumeration in fermented foods was previously described by Nero et al. (2006), but it is well known that MRS media does not provide proper selectivity for LAB, since non-LAB microorganisms can develop colonies in this medium (Kang & Fung, 1998). The addition of phenylethanol and lactic acid to MRS medium resulted in an increase in the selectivity for LAB, since all strains tested could be appropriately recovered in KF and KFS media and the raw milk samples showed lower counts when tested with these media. However, the added supplements generate some interference for Petrifilm™ AC plates. Also, the lower correlation between the methodologies using KF and KFS media for *Lactococcus lactis* subsp. *lactis* (r=0·42 and r=0·17, respectively – data not

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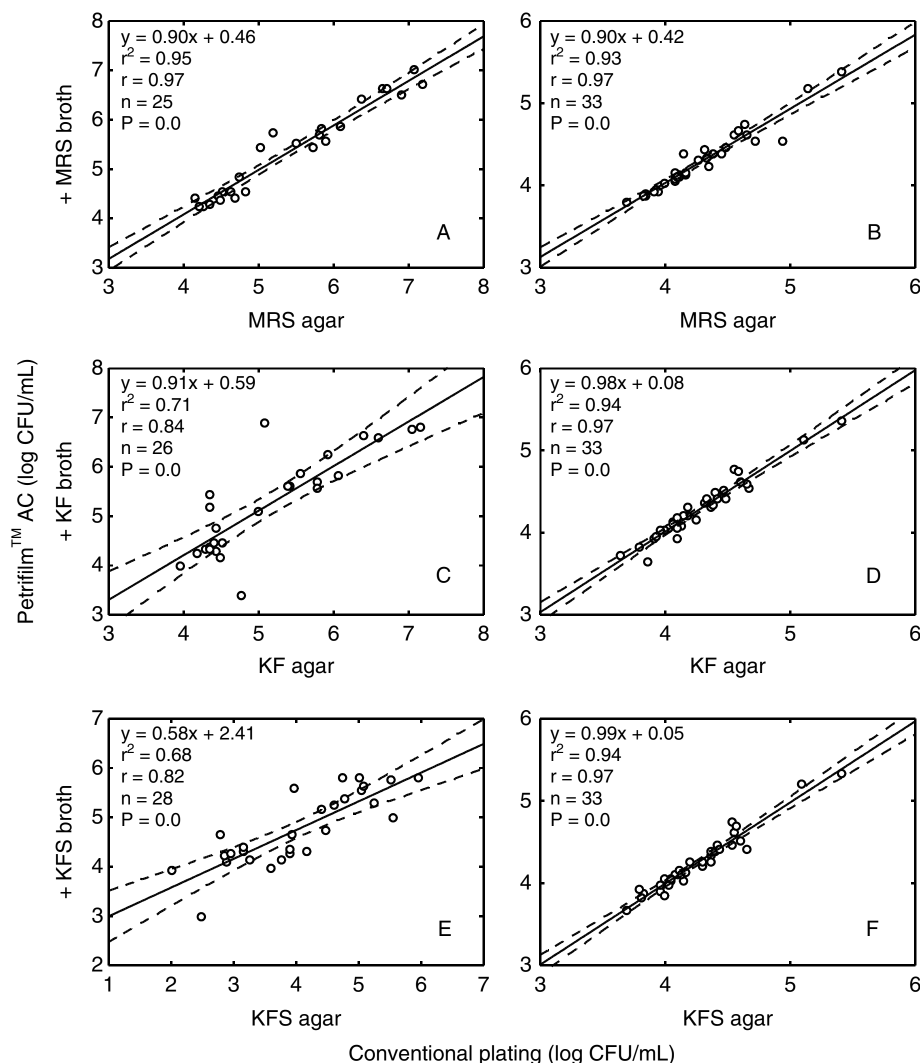

Fig. 1. Relationship between counts of microorganisms in raw milk (left) and Lactic Acid Bacteria cultures (right) by conventional methodology and Petrifilm™ Aerobic Count (AC), using de Mann-Rogosa-Sharpe (MRS, graphs A and B), Kang-Fung (KF, graphs C and D), and Kang-Fung-Sol (KFS, graphs E and F). In each graphic: r = correlation index, r^2 = determination coefficient, n = number of repetitions, P = level of significance.

shown) could be a probable reason for the same low correlation in raw milk samples. This microorganism is an important component of this product microbiota (Zamfir et al. 2006) and has already shown a poor recovery performance in MRS with these supplements (Kim et al. 2001).

The small size of the colonies from LAB cultures that grew on KF and KFS, when compared with MRS, can be considered as an indication of the selective activity of the supplements. Especially in KFS, the colonies showed a smaller size when compared with those on MRS and KF media, showing the inhibitory activity especially of the lactic acid. Even with this evident selectivity, all LAB strains could be properly recovered in this media. LAB are known as acid tolerant (Muyanja et al. 2003), and the pH 5·5 used in this study was sufficient to allow their growth and recovery. Zamfir et al. (2006) used a low pH to select *Lactococccus* spp. and *Lactobacillus* spp. in dairy products.

Although the enumeration of all strains tested in Petrifilm™ AC was possible, some of them showed almost transparent colonies in these plates. This particularly occurred with *Leuconostoc lactis* and *Streptococcus thermophilus* strains, and had already been observed by Nero et al. (2006) in a previous study. Beloti et al. (1999, 2002) reported that the performance of Petrifilm™ AC plates for enumeration of aerobes in Brazilian pasteurized milk was not good as described in the literature due to the presence of high levels of thermoduric species that are unable to reduce the dye (2,3,5-trypheniltetrazolium chloride) used as indicator in these plates. The inability of some bacterial strains to form visible colonies in

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Petrifilm™ AC was described by Dawkins et al. (2005) who related this deficiency of *Streptococcus* spp., as observed in this study.

KFS could be used as a selective media for screening LAB in foods, including raw milk, as proposed by Kim et al. (2001). However, a descriptive study of the different microorganism genera that can be recovered in each culture media and methodology must be conducted to confirm the selectivity of KF and KFS, associated or not to Petrifilm™ AC, for LAB screening.

Petrifilm™ plates are well known for being an important tool for control of quality in industry, optimizing hands-on work, space and costs in laboratories (Byrne & Bishop, 1991; Beuchat et al. 1998, Ferrati et al. 2005; Tavolaro et al. 2005). For LAB enumeration, the present study confirms the efficiency of Petrifilm™ AC plates, despite the supplements added to MRS broth.

The authors are thankful to 3M do Brasil Ltda. for providing the Petrifilm™ AC plates used in the study. Also to Juliana Scombatti Martins for the grammar and spelling review.

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