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Evaluation of Petrifilm™ system compared with traditional methodology in count of indicators of sanitary-hygienic quality and pathogenic microorganisms in sheep milk

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Abstract

The objective of this study was to evaluate the applicability of the Petrifilm™ plates to enumerate microbial groups in sheep milk. Samples of sheep milk (n = 30) were plated simultaneously, to enumerate mesophilic aerobes, total coliforms, lactic acid bacteria, *Staphylococcus aureus* and *Escherichia coli*, using convencional reference protocols and Petrifilm™ plates. The results were compared using McNemar's test, linear regression and ANOVA (p < 0,05). The results demonstrated good significant between conventional methodologies and Petrifilm™ plates. Further, the Petrifim™ STX for counting *S. aureus* had higher recoverability of bacteria compared with the conventional methodology. Based on the results obtained and in view of the ease and rapidity procedures results, Petrifim ™ plates may be considered as alternatives for microbiological testing in sheep milk.

Keywords: milk analyzes; sheep's milk; *Escherichia coli*; Aerobic mesophilic bacteria.

Practical Application: This study generated knowledge for the practical application of the Petrifilm™ system in microorganisms search in sheep milk. From this study was possible to conclude that the use of this system is reliable for microorganisms surveyed, facilitating the work, and generate faster results, an important factor when it comes to monitoring of food-borne outbreaks. Thus, it was possible to verify the adequacy of Petrifilm™ system as a new alternative for enumeration of mesophilic aerobic microorganisms, coliforms, lactic acid bacteria, *Staphylococcus aureus* and *Escherichia coli*. Finally, the use of Petrifilm™ system generated reliable results more easily and more quickly, including facilitating the development of the work in the laboratory microbiological analyzes of food, because of no necessity of using large quantities of materials, glassware, petri plates, pipettes, while minimizing the preparation of material for the analyzes.

1 Introduction

The microbiological quality of milk is a very broad and generic term. The main microorganisms involved in milk contamination are bacteria, viruses, fungi and yeasts. The absence of realization of the physico-chemical, enzymatic and microbiological analyzes difficult to evaluate the quality of pasteurized milk, prevents the rapid identification and immediate correction of the probable processing failures. Therefore, it is extremely important the milk analysis in order to establish a constant monitoring to ensure the quality of the product that will be consumed (Zocche et al., 2002).

The traditional microbiological analyses used in aliments quality control were developed in the end of the XIX century and still have been used up to now. However, those methodologies require a great time availability and excessive laboratory work (Silva et al., 2006). The traditional methodologies limitations have ended up contributing to the development of alternative aliments microbiology methodologies.

Several techniques to enumerate and identify bacteria have been studied. Nowadays they have been named fast methods, because they are more practical and simple in their execution, they require less material quantity and provide faster results. (Freitas et al., 2009; Beloti et al., 2002; Nero et al., 2000).

Petrifilm™ is composed by a double film system: on film is the basis and it is covered by dehydrated nutrients and gelling agents soluble in cold and a superior polyethylene film that contains indicators and gelling agents. The sample, diluted or not, is inoculated on the base film surface and the superior film is overlaid. The sample is spread in a certain area with the help of a plastic diffuser. After the solidification of the gelling substance the set is incubated at the time and temperature indicated by the manufacturer. After the incubation, the colonies are enumerated and the result is expressed in UFC/mL (Nero et al., 2000).

Petrifilm™ system has been highly accepted as an alternative to the depth sowing of plates counting standard methods in the aliments microbiological analysis. The Petrifilm™ plates for counting of aerobic mesophile microorganisms and coliforms in milk and its dairies are recognized by the Association of Official Analytics Chemists and Standard Methods for Examination of Dairy Products (Nero et al., 2000). Those methods can

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also offer space and material saving increasing the laboratory productivity (Beloti et al., 2002).

Researches about ready to use systems performance, like Petrifilm™ for sheep milk analyses are not found. Therefore, this study evaluated this system's performance as an alternative to traditional methods in sheep milk samples.

2 Material and methods

30 milk samples from Santa Inês sheep breed were collected for this survey on alternated days. All samples were collected in sterilized, identified and sample bottles and were kept on refrigeration during the transportation until the moment of the analyses.

The microbiological analyses for the comparison with the conventional and fast method were done simultaneously for the evaluation of the aerobics mesophilic microorganisms (AM) and total coliforms (CT) *Escherichia coli* (EC) *Staphylococcus aureus (SA)* and lactic acid bacteria (BAL).

Serial decimal dilutions in saline solution 0.85% (m/v) were done from every whole milk sample after a complete homogenization for AM, CT, EC and SA enumeration. For BAL counting, Man-Rogosa-Sharpe broth was used (MRS, Oxoid Ltd., Basingstoke, England) as diluent, according to Nero et al. (2006) protocol. For the AM ,CT, EC and SA survey, all the protocol found in the Normative Instructions number 62, from August 23rd, 2003, from Agriculture, Livestock and supply Ministry (Brasil, 2003) was followed, according to the conventional method. For the AM counting, 1.0 mL of every selected dilution duplicated in sterile Petri plates was seeded and added from 15 to 20 mL of standard counting Agar (PCA). After the solidification, the plates were incubated inverted to 36 +/−1°C for 48 hours. The colonies were enumerated and the obtained results were expressed in Forming Colonies Units/mL.

In SA enumeration, 0.1 mL of every selected dilution was inoculated on Agar Baird –Parker (BP) dried surface. The plates were incubated inverted to 36 +/−1°C from 30 to 48 hours. Typical colonies (T) were enumerated: Opaque ring Shiny black, surrounded by a clear transparent halo highlighted over the opacity from the environment; and also atypical colonies (A): grey or black shiny ones, with only one of the halos or no halos. After this process, coagulase, catalase and Gram coloring tests were taken. The final result was obtained by the sum of the results of typical and atypical confirmed colonies and expressed in UFC/mL.

For the CT counting, three dilutions were selected and seeded in duplicate in Violet Red Bile Agar (VRBA), inoculating 1.0 mL in plates containing 15 mL of VRBA previously molten. After the total environment solidification, it was added to every plate an over layer of 10 mL of VRBA previously molten and kept on 46-48°C in water bath. After complete solidification of the environment, the plates were incubated inverted to 36 +/−1°C for 18 to 24 hours. The coliforms typical morphology colonies were enumerated, that is, the rose colonies, containing from 0.5 to 2 mm diameter, surrounded or not by a bile precipitation zone present in the environment, and the atypical colonies. And then, from three to five colonies from each one, were submitted to confirmatory tests in CVBBL and EC broth.

The CT confirmation was obtained by the inoculation of every selected typical and atypical colonies in tubes filled with 2% lactose Bile shiny green broth (CVBBL) and incubation to 36 +/−1°C for 24 to 48 hours. The obtained results for each colony, well as the used dilution, were written down. The confirmation of thermotolerant coliforms (CTt) presence was done by the inoculation of 0.3 mL rate of every positive sample in CVBBL, in tubes filled with 4.0 ml of EC broth and later incubation to 45 +/−0.2°C for 24 to 48 hours in water bath. The results were expressed in UFC/ml.

The enumeration of acid lactic bacteria either in conventional methods or in Petrifilm™ use, was based on the protocol described by Nero et al. (2006) and Nero et al. (2008). From three selected dilutions, 1.0 mL was inoculated in Agar MRS (Wehr & Frank, 2004) and 1.0 ml in Petrifilm™ AC plate. The MRS plates were placed in anaerobiosis bottles with microaerophilic generators (Anaerobe Container System, Gaspak™ EZ, BD) and incubated at 35°C for 48 hours and the Petrifilm™ AC ones, incubated in 30°C for 72 hours also in anaerobiosis. Then, the Agar MRS plates containing 25-250 colonies were selected and the colonies were enumerated. The results were expressed in Colonies Forming Units/mL/g (UFC/mL/g).

As a fast method, AC, EC and STX Petrifilm™ system was used for AM, CT, EC and *Staphylococcus aureus* counting respectively, according to the manufacturer's instructions: with the pipette perpendicularly positioned toward Petrifilm™ plate, 1.0 mL of every wanted dilution was inoculated in the centre of the inferior film, and the superior film was carefully positioned adequately to avoid air bubbles formation. Indicated diffusers for every kind of plate were used to distribute the inoculum in the area, according to the instructions. The plates were incubated for 24-48 hours at 35°C +/−1°C and the results expressed in UFC/mL.

The obtained data from the counts was adjusted according to the dilutions and analyzed by qualitative test using descriptive statistics. For SA, CT, EC and BAL, McNemar's test was used to compare the samples frequencies over 1 UFC/mL for coliforms, 1 UFC/mL for *Escherichia coli,* 10 UFC/mL for thermo-nuclease positive *staphylococcus aureus*, and 1log UFC/mL for acid lactic bacteria and verification of correlation between conventional methods and Petrifilm™ system.

For some groups, it was verified a coincidence in the results for McNemar ($P < 0.5$). The AM counts were converted in log and methods compared by linear reduction ($P < 0.05$). The software XLSTAT 2010.2.03 was used for the whole analysis.

3 Results e discussion

From 30 analysed samples, 27 were taken into consideration in the statistic evaluation for aerobic mesophilic, showed that there was no significant difference among the compared averages between both methods, according to Table 1 and, meaningful correlation of 0.942 (Table 2). The Figure 1 shows dispersion of mesophilic aerobics counting data, proving the correlation between both methods.

Carvalho et al. (2002) when evaluating the Petrifilm™ system as an alternative for traditional methods for mesophilic aerobics count in refrigerated raw milk, was obtained a correlation of 0.9682 and suggested a substitution of conventional methods of refrigerated raw milk microbiological control for Petrifilm™ system.

Freitas et al. (2009) when evaluated official protocols for the enumeration of mesophilic aerobics in raw and pasteurized milk, they concluded that both methods can be used.

Rosmini et al. (2004) obtained a correlation index of 0.92 and concluded that Petrifilm™ system is a valid alternative for mesophilic aerobic micro-organisms enumeration in raw milk compared to conventional techniques.

With regard to BAL, when the frequencies of negative and positive results were compared, it was verified that the methods present a good combination of coincident results and a 0.344 p value. Moreover, it was observed that Petrifilm™ AC when used in the specific fast method to BAL, is able to recover a bigger number of colonies (Table 3).

It is also taken into consideration the hypothesis of some samples might have presented false-negative results in the conventional method by the comparison of the results because in the negative combination for the conventional method and positive combination for Petrifilm™ AC, seven sample frequency was obtained (Table 3).

It was also obtained some low counts of BAL and in 11 samples there was no colonies growth in both methods even when they were entirely seeded, without previous dilutions. It probably happened because the samples were collected straight from the sheep teats after the hygienization and elimination of the first milk jets and kept under refrigeration. It means that there was no environmental or temperature interference where the samples are kept stored.

Nero et al. (2006), when compared Petrifilm™ AC and Agar MRS (Man-Rugosa-Sharpe) for the enumeration of BAL in fermented milk, it was observed that the difference in both methods was not significant, with a significance level of 0.05, independent from the BAL type tested. Also, Nero et al. (2008) when compared the performance of Petrifilm™ AC for BAL count in fermented milk observed a high correlation index and no significant differences in both methods.

After the count analysis of *Staphylococcus aureus,* it was observed in results comparison, that the combination negative for conventional method and positive for Petrifilm™ STX obtained a frequency of 12 samples. It may mean the Petrifilm™ STX has a larger capacity of recover more colonies in comparison with the conventional method.

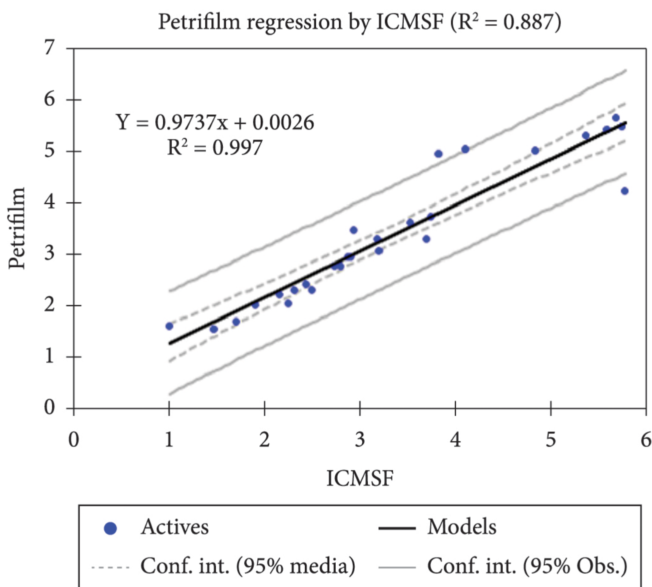

Figure 1. Dispersion of mesophilic aerobics count data from raw sheep milk samples, obtained by conventional method (Brasil, 2003) (x) and with Petrifilm™ plates AC (y).

Table 1. Mesophilic aerobics (logUFC/mL) average values count (± standard deviation) from raw sheep milk samples obtained from conventional methods (ICMSF) and using Petrifilm™ AC plates.

Method	Samples	N	Mesophilic aerobics Average (DP)
ICMSF	30	27	3.34 ± 1.38
Petrifilm™	30	27	3.38 ± 1.32
ANOVA – F(1.25) = 195.64; P < 0.05			

F: ANOVA value. P: significance level. N: number of used samples.

Table 2. Correlation parameters among mesophilic aerobics counts (log UFC/mL) from sheep milk obtained by conventional method (Brasil, 2003) and using Petrifilm™ AC plates.

Group	Samples	n	R	r^2	Equation	a	b	p
mesophilic aerobics	30	27	0.942	0.89	y=0.38+0.89*x	0.89	0.38	<0.05

n: number of samples with matched results between the two methods. R: correlation index.r^2: determination coefficient. determination of regression, where y= Petrifilm™ e x=ICMSF. a: inclination of regression straight line. b: intercept of regression straight line. p: significance level.

Table 3. Comparisons in frequencies in negative and positive results for coliforms presence (> 1UFC/mL), *Escherichia coli* (> 1UFC/mL) S*taphylococcus aureus* positive coagulase (> 10UFC/mL) and acid lactic bacteria (> 1log UFC/mL) in raw sheep milk samples analysed by conventional method (ICMSF) and Petrifilm™ system.

Group	Combination (ICMSF:Petrifilm)	Coliforms	*E.coli*	*S.aureus*	BAL
Coincidents	positive:positive	4	0	3	7
	negative:negative	24	30	14	10
Divergents	positive:negative	2	0	1	3
	negative:positive	0	0	12	7
Statistics		p = 0.50 Q = 2.0	p = 1.00 Q = 0.0	p = 0.003 Q = 9.3	p = 0.344 Q = 1.6

Value of p <0.05 indicate significant differences between the methodologies compared. Q - McNemar Test.

Santos (2009), when compared Baid-Parker and Petrifilm™ STX culture media in the detection of *Staphylococcus* positive coagulase in naturally contaminated raw milk and sterilized milk inoculated with specific cultures, it was verified a statistically significant difference in both methods. According to the author, this fact may be attributed to the presence of a concomitant high microbial quantity in the samples that although doesn't get developed in the environment due to the action of inhibiting substances it contributes to the *Staphylococcus* spp. growth. Regarding the sterilized milk inoculated in specific cultures, realized by the results presented, the two methods were effective in detecting *Staphylococcus* spp. In this research, high quantities of *S.aureus* (2.370 UFC mL. average) in some samples what proves that under the condition those collections were done, this high quantity of *S.aureus* can be related to infections in th animals mammary glands sample.

Ingham et al. (2003) When compared Agar Baird-Parker and Petrifilm™ STX for the enumeration of *S.aureus* in naturally or artificially contaminated aliments, it was concluded that there wasn't a significant difference on methods for naturally contaminated raw milk.

Viçosa et al. (2010) likewise, it was observed no significant differences between Agar Baird-Parker and Petrifilm™ STX in the enumeration of positive coagulase and thermo-nuclease *S.aureus* in raw milk and fresh cheese samples.

Silbernagel et al. (2003) also reported a similar result when evaluated Petrifilm™ STX in dairy aliments, where raw milk and mozzarella cheese also presented good results of correlation index between fast and conventional methods.

About the enumeration of total coliforms and *E.*coli/thermo-tolerant coliforms, the results were coincident between both methods, as shown on Table 3. In most of the samples (27) the quantity of these micro-organisms remained very low or null in both methods and when they presented some counts, they were coincident.

Carvalho et al. (2002) when evaluated Petrifilm™ as an alternative to traditional methods of total coliforms count in refrigerated raw milk, its use was suggested when a correlation of 0.8380 was obtained.

The use of Petrifilm™ has been suggested as an alternative method for the enumeration of these micro-organisms, reaching international acceptance due to its practicality on its execution and the reduction of time in results obtainment (Ponsano et al., 2000) and effectiveness in the indication of fecal origin contamination through *E.coli* direct identification.

4 Conclusion

The obtained results proved a good correlation among conventional methods and Petrifilm™ system for microbiological analyses of mesophilic aerobics, total coliforms *Escherichia coli, Staphylococcus aureus* and acid lactic bacteria in sheep milk. In addition to, Petrifilm™ STX presented a bigger capacity of bacteria recovering when compared to conventional methods.

Based on the obtained results and taking into consideration a great facility in procedures and rapidity in results, the Petrifilm™ system can be used in microbiological analyses in sheep milk.

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