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Research Paper

# Evaluation of the Petrifilm™ EB and TEMPO® EB systems with ISO 21528-2:2004 method for the count of *Enterobacteriaceae* in milk

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## Abstract

The development of alternative microbiological techniques is driven by the necessity to meet the current needs to deliver rapid results in the manufacturing process of foods, but it is important that these methods be evaluated for each application. The objective of the present study was to assess the Petrifilm™ EB and the TEMPO® EB systems with ISO 21528-2:2004 for the count of *Enterobacteriaceae* in pasteurized and UHT milk samples. We analyzed the microflora of 141 pasteurized milk samples, 15 samples of artificially contaminated pasteurized milk and 15 samples of artificially contaminated UHT milk. Investigation of the method Petrifilm™ EB and ISO 21528:2 regression analysis showed a high correlation in the samples, r = 0.90 for the microflora of pasteurized milk, r = 0.98 for artificially contaminated pasteurized milk and r = 0.99 for the artificially contaminated UHT milk. In evaluating the system TEMPO EB ® method and ISO 21528:2 correlation was also significant in the analyzed samples, with r = 0.86 for the microflora of pasteurized milk, r = 0.96 for artificially contaminated pasteurized milk and r = 0.99 for artificially contaminated UHT milk. No statistically significant differences were observed between the three methods conducted to analyze artificially contaminated pasteurized and UHT milk at three inoculum levels. In conclusion, the Petrifilm™ EB system and the TEMPO® EB system may be an alternative to the ISO 21528-2:2004 for the *Enterobacteriaceae* assay for milk as because of the ease-of-operation and the time reduction achieved for conducting the microbiological assay using these systems.

**Key words:** *Enterobacteriaceae,* milk, alternative methods, ISO 21528-2:2004, count.

## Introduction

The *Enterobacteriaceae* are a large family of Gram-negative, rod-shaped, facultative anaerobic bacteria. They have been used for many years as microbiological quality indicators and food safety index organisms in Europe. The presence of *Enterobacteriaceae* generally indicates problems with food processing hygiene, such as inadequate heat treatments or post-processing contamination from raw materials or the environment (Kornacki and Johnson, 2001; Owen *et al.*, 2010).

In the European Union and in other developed nations, the *Enterobacteriaceae* test is a mandatory analysis for many types of food products. On the other hand, in Brazil, one of the largest producers of milk and dairy products, food regulations do not require the *Enterobacteriaceae* test for these foods, being only the analysis of Coliforms and *Samonella* sp mandatory. It makes it difficult for the insertion capability of Brazilian products in the global market, which is dependent on the international standards, such as the microbiological quality (Ferraz *et al.*, 2010).

Traditionally, methods for count of bacteria by plating into or onto agar have been used for microbiological

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evaluation. However, new methods based on the technology of chromogenic and fluorogenic indicators have been developed to serve as alternative to the conventional methods. These new methods deliver not only faster results, economy of space and materials, but also increase laboratory productivity as compared to the conventional techniques (Beloti *et al.*, 2003; Casarotti *et al.*, 2007).

One of the alternative methods employed for counting *Enterobacteriaceae* in foods is the Petrifilm™ EB system (3M Company, St. Paul, MN, EUA) - a ready-to-use system consisting of a double-film system. The bottom film is covered with dehydrated nutrients and a cold-water-soluble gelling agents, whereas the top film contains gelling agents and a color indicator. The Petrifilm™ EB plate is a rapid alternative for performing *Enterobacteriaceae* counts using Violet Red Bile Glucose as culture medium (VRBG) and tetrazolium red as color indicator (TTC), with incubation at 35 °C for 24 hours (Nero *et al.*, 2000; Kornacki and Johnson, 2001; Jasson *et al.*, 2010).

Another alternative method is the TEMPO® (Biomérieux S.A.) system, one of the most recently developed automated count systems for indicators of the microbiological quality of foods. The system consists of a card and a vial containing the dried culture medium and a fluorescent indicator. The inoculated medium is automatically transferred by TEMPO® Filler to a card containing three levels of 16 wells each, with the volume of each well of the first row being 225 μL, 22.5 μL being the volume of the wells of the second row and 2.25 μL that of the third row. The card is incubated for 24 hours at 35 °C (TEMPO® EB) and after the reading is performed by the TEMPO® Reader based on the fluorescence which calculates the number of positive wells and expresses the results in CFU $mL^{-1}$. The TEMPO® EB system is based on the fermentation of glucose by the *Enterobacteriaceae*, which causes acidification of the reagent and results in the extinction of the fluorescence in the tubes that tested a positive reaction. (Torlak *et al.*, 2008; Owen *et al.*, 2010).

However, due to interference of regional variations in microflora or the food matrix, comparative investigations between conventional methods of analysis should be conducted, since very often intrinsic characteristics of foods may interfere with the results produced by these alternative methods (Casarotti *et al.*, 2007).

Based on these considerations, the objective of this study was to compare the Petrifilm™ EB system (3M Company, St. Paul, MN, EUA) and the TEMPO® EB system (Biomérieux S.A.) with ISO 21528-2:2004 in performing *Enterobacteriaceae* counts in samples of pasteurized and UHT milk.

## Material and Methods

### Milk samples

A total of 156 samples of pasteurized milk and 15 samples of UHT milk, all produced under the supervision of the Brazilian Federal Food Inspection Service (SIF) in the State of Santa Catarina were collected from different dairy plants located throughout the State of Santa Catarina, Brazil. The samples were collected and stored in iceboxes containing recyclable ice to keep the temperature below 4 °C (Brasil, 2002). Next, they were transported to the Microbiology Laboratory of the Department of Food Science and Technology of the Federal University of Santa Catarina.

The contents of the packages was homogenized by manual agitation. The packages were cleaned with cotton and disinfected with 70% alcohol. After opening, 9 mL volumes were withdrawn from each package and transferred to sterile tubes. These samples were then diluted with 0.1% peptone water (British Standards Institution, 1999).

### Artificial contamination

15 pasteurized milk samples and 15 UHT milk samples randomly selected were artificially contaminated. These samples were divided into two groups, one group was contaminated with a culture of the *Enterobacteriaceae* family (*Salmonella Typhimurium* ATCC 14028) and another group with a negative control culture, a strain of *Staphylococcus aureus* (ATCC 02923). The cultures were reactivated in nutrient broth (Oxoid, Ltd, Basingstoke, UK) and incubated at 37 °C for 18 h (Owen *et al.*, 2010). The samples were adjusted by the dilution technique in 0.1% peptone water, in order to obtain three different inoculum levels, in accordance with the procedure described by Feldsine *et al.* (2002). The milk samples were divided over four groups. The first group (five samples) was inoculated with $10^1$ cfu $mL^{-1}$ (low level), the second (five samples) with $10^2$ cfu $mL^{-1}$ (medium level), the third group (five samples) with $10^3$ cfu $mL^{-1}$ (high level). A fourth group was left un-inoculated to serve as negative control sample, and used to calculate the difference between artificial contamination and microflora of the milk, in a way such that would express only the value of the inoculum added at the different levels investigated.

### Analysis

We analyzed the microflora of 141 pasteurized milk samples, 15 samples of artificially contaminated pasteurized milk and 15 samples of artificially contaminated UHT milk by method ISO 21528-2:2004, Petrifilm™ System and TEMPO® System. All analyzes were performed in duplicate.

### Microbiological techniques

#### *ISO 21528-2:2004*

1.0 mL aliquots of each of the dilutions were transferred to Petri plates and added with about previously melted 10 mL of the Violet Red Bile Glucose agar culture medium (VRBG - Oxoid Ltd., Basingstoke, Hampshire, England), and kept at 44 °C - 47 °C in a water bath. The

inoculum was carefully homogenized with the culture medium until total solidification. Next, a second layer (15 mL) of the same culture medium was added and left to stand until complete solidification and followed by incubation at 37 °C for 24 ± 2 hours. Once this period had elapsed, the pink-to-red-to-purple colored colonies were counted, surrounded or not by a precipitation halo. Five colonies were transferred and sub-cultured on plates with Nutrient Agar (AN- Oxoid Ltd., Basingstoke, Hampshire, England) (37 °C for 24 ± 2 hours) for further confirmation with the oxidase and glucose fermentation tests, being confirmed as oxidase-negative and glucose-positive *Enterobacteriaceae* (British Standards Institution, 2004).

### *Petrifilm™ system*

Using the Petrifilm™ system (3M Company, St. Paul, MN, EUA), the EB (*Enterobacteriaceae*) plates were inoculated with 1.0 mL of each of the dilutions and gently and evenly spread with a plastic spreader over the plate growth surface of the 20 $cm^2$ plates. The plates were incubated with the transparent side facing upward in a laboratory incubator at 35 °C for 24 hours. According to the instructions of the manufacturer, red colonies with or without gas production and/or yellow zone are considered positive for *Enterobacteriaceae* (Kornacki and Johnson, 2001).

### *TEMPO® system*

Aliquots containing different levels of inoculum were transferred to TEMPO® EB flasks (Biomérieux S.A.). These aliquots were diluted and automatically transferred to a card containing 48 wells of three different volumes (16 x 225, 16 x 22.5, 16 x 2.25 µL), using TEMPO Filler®. After incubation for 24 hours at 35 °C, the results were read on the TEMPO Reader®. Once the reading of the results was concluded, they were automatically analyzed by the *software* system that determines which of the wells tested positive. The number of positive wells obtained in relation to the volume of wells and the dilution of the samples, automatically allows the enumeration of the results in CFU $mL^{-1}$, based the Most Probable Number (MPN) tables (AOAC Research Institute, 2011).

## Statistical analysis

The values obtained were converted to logarithmic form, and the results subsequently subjected to regression analysis. The data of artificial contamination were subjected to analysis of variance (ANOVA). All statistical analyses were performed using the Statistica® 8.0 Software program (Statsoft, Inc, Statistica, 2004).

For the purpose of statistical calculation, whenever the results obtained were < 1 CFU $mL^{-1}$, these results were replaced by the number immediately below, that is, 0.9 CFU $mL^{-1}$.

## Results

It can be observed that there was a positive relationship between the different methods used for the enumeration of *Enterobacteriaceae,* as shown by the correlation coefficient (r), which demonstrated a positive linear association between the methods, as can be seen from Table 1. The countings microflora of UHT milk were not shown in Table 1, since all samples produced < 1 CFU/mL.

With regard to the evaluation of the artificial contamination with different levels of inoculum, (low, medium, high), the data were subjected to analysis of variance (Table 2). The results indicated that there was no statistically significant difference between the three methods investigated (ISO 21528-2:2004, Petrifilm™ EB system and the TEMPO® EB) system for counts *Enterobacteriaceae*, both in the pasteurized as in the UHT milk, at the three inoculum concentrations ($10^1$, $10^2$, $10^3$ cfu $mL^{-1}$) artificially added with a strain of *Salmonella Typhimurium.*

The results also show that the three methods analyzed for the count of *Enterobacteriaceae* did not identify at the three inoculum levels ($10^1$, $10^2$, $10^3$ cfu $mL^{-1}$) any count of the presence of the strain of *Staphylococcus aureus* added to the pasteurized and UHT milk (Table 2).

## Discussion

The correlation coefficients yielded by the analysis of the Petrifilm EB system with ISO 21528-2:2004 (Table 1)

**Table 1** - Correlation coefficient between ISO 21528-2:2004 and the Petrifilm™ EB and the TEMPO® EB in the enumeration of Enterobacteriaceae obtained by the microflora and artificially contaminated pasteurized milk and artificially contaminated UHT milk.

| Samples | (N)* | ISO 21528-2:2004 x Petrifilm™ EB r** | ISO 21528-2:2004 x TEMPO® EB r** |
|---|---|---|---|
| Microflora of the pasteurized milk | 141 | 0.90 | 0.86 |
| Artificially contaminated pasteurized milk | 15 | 0.98 | 0.96 |
| Artificially contaminated UHT Milk | 15 | 0.99 | 0.99 |

*(N) = number of samples of milk analyzed.
**r = correlation coefficient.

**Table 2** - Average counts in log CFU mL$^{-1}$ of Enterobacteriacea and those of a strain not belonging to the Enterobacteriaceae family yielded by ISO 21528-2:2004, Petrifilm EB and TEMPO® EB at different inoculum levels artificially added to pasteurized and UHT milk.

| Strain | Inoculum level*** | ISO 21528:2 | Petrifilm EB | TEMPO® EB |
|---|---|---|---|---|
| | | | System | System |
| | | Pasteurized | UHT | Pasteurized |
| Enterob** | Low | 1.41a* ± 0.06 | 1.23b ± 0.10 | 1.57a ± 0.26 |
| | Medium | 2.52a ± 0.15 | 2.54b ± 0.16 | 2.59a ± 0.14 |
| | High | 324a ± 0.15 | 3.18b ± 0.16 | 3.37a ± 0.08 |
| Non Enterob** | Low | < 1 | < 1 | < 1 |
| | Medium | < 1 | < 1 | < 1 |
| | High | < 1 | < 1 | < 1 |

*Means followed by the same letters, in the same row and type of milk did not present any statistical difference at the $p < 0.05$ level of significance.
***Enterobacteriaceae*: *Salmonella Typhimurium* (ATCC 14028), Non *Enterobacteriaceae*: *Staphylococcus aureus* (ATCC 02923).
***Inoculation level: low ($10^1$ cfu mL$^{-1}$), medium ($10^2$ cfu mL$^{-1}$), high ($10^3$ cfu mL$^{-1}$).

show a high correlation in counting *Enterobacteriacea* of microflora pasteurized milk samples, artificially contaminated pasteurized milk and artificially contaminated UHT milk.

Ferraz *et al.* (2010) also obtained a similarity between the Petrifilm EB system and ISO 21528:2 in the enumeration results of *Enterobacteriaceae* in a powdered milk production chain, after having analyzed raw milk, pasteurized milk, milk powder, equipment swabs and hand swabs.

The results relative to the correlation between ISO 21528-2:2004 and the TEMPO® EB system also showed a high correlation the counts of *Enterobacteriacea* of microflora pasteurized milk samples, artificially contaminated pasteurized milk samples and artificially contaminated UHT milk, as can be seen in Table 1.

Lower correlation coefficients were found by Owen *et al.* (2010), when they evaluated 209 samples of foods and dairy products using the TEMPO® EB system, the MPN method, the pour plate and the spread plate techniques, recording correlation coefficients of 0.78, 0.75 and 0.78 respectively.

Paulsen *et al.* (2008) evaluated the TEMPO® EB and Petrifilm ™ EB systems and ISO method 21528-2 in 411 naturally contaminated foods and their results did not present any significant difference between the methods, with means and standard deviation values of 2.540 ± 1.026, 2.547 ± 0.995 and 2.456 ± 1.014 log UFC/g for ISO 21528-2, Petrifilm ™ EB and TEMPO® EB, respectively.

It may also be observed in Table 1 the correlation coefficient obtained from the analysis of microflora pasteurized milk and artificially contaminated pasteurized milk obtained similar results in the correlation analysis (high correlation coefficients), showing a good performance of alternative methods analysis with pasteurized milk.

Evaluation of artificial contamination with different levels of inoculum ($10^1$, $10^2$, $10^3$ cfu mL$^{-1}$) of a strain of *Salmonella Typhimurium* in pasteurized and UHT milk, indicated that there were no statistical differences between the three methods analyzed (ISO 21528-2:2004, the Petrifilm EB system and the TEMPO® EB system) at each level of inoculum in the *Enterobacteriacea* counts. These results demonstrate that the alternative methods presented good identification results in environments of low, medium and high contamination.

A different result was reported by Silbernagel and Linderberg (2003), who in a study on artificial contamination with *Proteus vulgaris* at different inoculum levels in milk samples evaluated the performance of the Petrifilm EB system and compared the results to those obtained with the plate count method and the Most Probable Number (MPN) technique. In this study, the results showed that there was no statistical difference between the methods at an medium level of inoculation ($10^3$ CFU mL$^{-1}$). However, as for low ($10^2$ CFU mL$^{-1}$) and high inoculation concentrations ($10^4$ CFU mL$^{-1}$) they did exhibit a statistical difference, with the Petrifilm EB system presenting the highest mean value.

It may also be observed that the methods for the detection of *Enterobacteriaceae* were species-specific, *i.e.* unable to detect counts a strain not belonging to the *Enterobacteriaceae (Staphylococcus aureus)* family. They also demonstrated sensitivity since they also detected counts at the different contamination levels tested.

The specificity and sensitivity of the Petrifilm EB system can be explained by the detection principle of the method, with the plates using Violet Red Bile Glucose Agar (VRBG) as culture medium and 2, 3, 5-trifeniltetrazolium (TTC) as color indicator. This colorant is colorless in the oxidized form, and turns red when reduced by the mitochondrial succinate-desidrogenase enzyme of the microorganism, imparted by the formation of the formazan precipi-

tate, which is a dark-colored product (Kornacki and Johnson, 2001).

As for the TEMPO® EB system, the culture used in this system contains a fluorescent molecule (4-methyl umbelliferone). The growth of the microorganism hydrolyzes the culture medium during incubation, - a reaction that is accompanied by an increase in the pH value caused by the fermentation of the carbohydrate, resulting in the extinction of the fluorescence in tubes with a positive reaction (Owen *et al.*, 2010).

Silbernagel and Lindberg (2002) also conducted a similar study with 174 samples of naturally contaminated and 120 artificially contaminated dairy and non-dairy products, inoculated with three different inoculum levels (low, medium and high) and 45 pure non-*Enterobacteriaceae* strains and 65 pure cultures of *Enterobacteriaceae*, with the aim of evaluating the Petrifilm™ EB system and compare it against the performance of the VRBG Standard Method. The results showed that the Petrifilm™ EB system had an equal or better performance than the VRBG standard method and showed to be sensitive with a recovery rate of 97% of the *Enterobacteriaceae* present and also selective with only 16% of the non-*Enterobacteriaceae* being capable to grow on the Petrifilm™ EB plate.

Owen *et al.* (2010) also analyzed the sensitivity and specificity of the TEMPO® EB system by testing *Enterobacteriaceae* strains and non-*Enterobacteriaceae* strains at two inoculum levels (low and high). They concluded that the TEMPO® EB showed to be sensitive and specific.

According to the results, the Petrifilm EB system and the TEMPO® EB system can both be used as a valid alternative to ISO 21528-2:2004 for the *Enterobacteriacea* assay in milk, based on the good correlation between the results, enhanced ease-of-operation and a reduction in the amount of time customarily required by these systems.

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