Original

Evaluation of the use of Petrifilm™ EB count plates for the enumeration of *Enterobacteriaceae* in poultry samples

Avaliação do uso de placas Petrifilm™ EB para a enumeração de Enterobacteriaceae em amostras de carcaças de frango

Tainá DREBES[1]*, Cláudia MAJOLO[2], Hans FRÖDER[1]

Abstract

A test that is rapid, simple, accurate, not expensive, gives rapid results, and is sensitive enough to detect low levels of microorganisms would be the most suitable for food industry routine laboratories, or even for a public health laboratories. A ready-to-use alternative, commercially available method is the Petrifilm™ EB method. The aim of this study was to evaluate whether there is a statistically significant difference between the conventional methods based on Violet Red Bile Glucose Agar and the alternative 3M™ Petrifilm (EB) method for the enumeration of *Enterobacteriaceae* in poultry carcasses. This study also assessed whether the alternative method showed ability to produce results that were directly proportional to the concentration of the target (approximately 270 colony-forming unit.mL^{-1}). A total of 120 poultry carcasses samples showed a significant difference ($p < 0.05$) between the populations obtained by the two methods, and the conventional method showed low proportionality between the dilutions**.** On the other hand, the Petrifilm™ EB quantification system showed the capacity to produce results that are proportional to the concentration of the analyte in samples in the concentration range from 1 to 256 colony-forming unit.mL^{-1}.
Keywords: *alternative method; linearity test; indicator microorganisms.*

Resumo

Um teste rápido, que apresente o resultado em curto período de tempo, que seja simples, sensível para detectar baixos níveis de micro-organismos, preciso e que não seja caro, seria o mais adequado para a rotina laboratorial da indústria de alimentos ou mesmo para os laboratórios de Saúde Pública. Um dos métodos alternativos prontos para o uso, comercialmente disponíveis no mercado, são as placas Petrifilm™. O objetivo deste estudo foi avaliar se há diferença estatística significativa entre o método convencional – empregando o ágar vermelho violeta bile glicose – e o método alternativo, Petrifilm™ EB (3M Company), para a enumeração de *Enterobacteriaceae* em carcaças de frango. Também foi avaliado se o método alternativo apresentou capacidade de produzir resultados que fossem diretamente proporcionais à concentração do alvo (contendo aproximadamente 270 unidades formadoras de colônia.mL^{-1}). O total de 120 amostras de carcaças de frango analisadas revelou que houve diferença significativa ($p < 0,05$) nas populações obtidas entre os dois métodos, sendo que o método convencional apresentou pouca proporcionalidade entre as diluições realizadas. Por outro lado, o sistema de quantificação Petrifilm™ EB mostrou capacidade em produzir resultados proporcionais à concentração do analito nas amostras, em uma faixa de concentração de 1 a 256 unidades formadoras de colônia.mL^{-1}.
Palavras-chave: *método alternativo; teste de linearidade; micro-organismos indicadores.*

1 Introduction

Enterobacteriaceae is a diverse family that includes a large number of disease-causing pathogens in man and animals. These organisms are related to the major causes of intestinal infections in different countries and are the main hospital infection agents (TRABULSI, 2005).

Enterobacteriaceae are Gram-negative bacilli and oxidase negative. They produce acid from glucose; are facultative anaerobes; reduce nitrate to nitrite; and may be motile or non-motile (KORNACKI; JOHNSON, 2001). Members of *Enterobacteriaceae* are commonly used as indicator microorganisms for assessing food safety and hygiene since they are found in the gastrointestinal tract of humans and animals (FORSYTHE, 2002) and can cause diseases and even economic losses (TRABULSI, 2005).

The presence of *Enterobacteriaceae* on poultry carcasses is similarly related to slaughter, processing, and storage. Among the kinds of bacteria found in poultry, the main representatives of the family *Enterobacteriaceae* include *Citrobacter*, *Enterobacter*, *Escherichia*, *Proteus*, *Salmonella*, and *Serratia* (JAY, 2005).

Laboratory methods currently used for microbiological examination of food can be classified as conventional and alternative. The former are named this way because they have been developed for a very long time (some of them for almost 100 years); have currently been used in food

Received 20/9/2011
Accepted 16/5/2012 (005477)
[1] *Food Microbiology Laboratory, Centro Universitário Univates – UNIVATES, Rua Avelino Tallini, 171, Building 5A, CEP 95900-000, Lajeado, RS, Brazil, e-mail: tainadrebes@univates.br*
[2] *EMBRAPA Amazônia Ocidental, Rod. AM-010, Km 29, Rural Zone, CP 319, CEP 69010-970, Manaus, AM, Brazil*
**Corresponding author*

DOI: http://dx.doi.org/10.1590/S0101-20612012005000088

microbiology laboratories and in reference publications; and are internationally accepted. In the conventional method, the enumeration of *Enterobacteriaceae* in foodstuff is performed using Violet Red Bile Glucose Agar (VRBG) (BRASIL, 2003; KORNACKI; JOHNSON, 2001).

From the laboratory perspective, the characteristics of the conventional methods include the need for glassware, culture media, reagents, incubators, and water baths. Additionally, they need a long time to obtain results, and there is a great possibility of errors during analysis or when reading and interpreting results (FRANCO, 1994). The method can be proprietary or non-commercial and does not need to cover the entire examination procedure, i.e., from sample preparation to final results. When choosing a rapid method, some factors such as ability to detect low levels of microorganisms, accuracy, precision, low cost, speed, ease of use, and international acceptance should be taken into account (INTERNATIONAL…, 2003).

One of the ready-to-use and commercially available alternative methods is the Petrifilm™ EB plate used specifically for the enumeration of *Enterobacteriaceae*. The Petrifilm™ EB method was approved by the Association of Official Analytical Chemists (ASSOCIATION…, 2006); it is described in the Compendium (KORNACKI; JOHNSON, 2001) and in the Standard Methods for the Enumeration of Dairy Products. Petrifilm™ EB is a modification of the Colony Forming Units (CFU) count on plates. A Petrifilm™ EB plate comprises two rehydratable sterile films impregnated with the culture medium and cold-water-soluble gelling agents.

There are few studies in the literature comparing the enumeration of *Enterobacteriaceae* in poultry samples using the Petrifilm™ EB method and the conventional method (VRBG). Silbernagel and Lindberg (2003) compared the use of the Petrifilm™ EB plates with the conventional method using cheddar cheese, milk, flour, frozen prepared meals, nuts, frozen broccoli, and pure cultures.

This study aims to evaluate whether there is a meaningful statistic difference between the data obtained by the conventional method and those obtained by the Petrifilm™ EB method when enumerating *Enterobacteriaceae* on poultry samples as well as to evaluate the linearity of the alternative method when using pure culture.

2 Materials and methods

A total of 120 poultry carcasses samples were collected in different cities and abattoirs in the state of Rio Grande do Sul from August to December 2009. The samples were analyzed for *Enterobacteriaceae* at the Food Microbiology Laboratory UNIANÁLISES, at Centro Universitário UNIVATES, in Lajeado/RS, when required.

Carcass sampling involved taking a total of 25 g using sterile scissors and placing them into individual stomacher bags. All samples were transported to the laboratory in an insulated box containing ice packs.

For the linearity test of the Petrifilm™ EB method, the reference strain *Enterobacter aerogenes* ATCC 13048-1, provided by the culture collection of the same lab, was used.

Following the conventional method described in the Normative Instruction Nº. 62 (BRASIL, 2003), appropriate dilutions of the sample were selected and inoculated on Violet Red Bile Glucose Agar (VRBG) (Becton, Dickinson and Company, USA). After complete solidification of the medium, an overlay medium was added using the Pour Plate technique. The plates were incubated upside down from 18 to 24 hours at 36 °C ± 1 °C.

Plates containing between 15-150 colonies (only typical colonies on VRBG agar, which were dark red stained, 0.5 mm or more in diameter, and surrounded or not by a halo of precipitation of bile salts present in the culture medium) were selected. For confirmation, 3-5 colonies were submitted to oxidase and Gram staining tests. Colonies that are oxidase negative and Gram-negative are typical of the family *Enterobacteriaceae*. The calculation was based on the number of populations obtained (respecting the limit of precision and repeatability) divided by the number of colonies tested and multiplied by the total colonies that were characteristic for the test. The results were expressed in $CFU.g^{-1}$.

Similarly to what is done in the conventional method, suitable dilutions were selected and 1 mL of each dilution was inoculated on the Petrifilm™ EB plates (3M, USA). The procedure followed the manufacturer's guidelines. After inoculation, the top film was gently rolled down on the inoculum preventing the formation of bubbles. It took 2-5 minutes for gelling to occur. The plates were incubated for 24 ± 2 hours at 35 °C ± 1 °C. Plates with 15-100 typical colonies were counted based on the three following characteristics: red with gas bubbles and no yellow halo, red with yellow halo and no gas bubbles, and red with yellow halo and gas bubbles. The population obtained was expressed in $CFU.g^{-1}$ by multiplying the number of typical colonies by the inverse of the dilution.

In Figure 1, it is possible to observe the schematic representation of the procedures used in both methods (conventional and alternative).

Based on the linearity, the Petrifilm™ EB method was evaluated for its ability to produce results that are directly proportional to the target concentration. A stationary phase containing a population of 270 $CFU.mL^{-1}$ was used to do it. An overnight culture of *Enterobacter aerogenes* ATCC 13048-1 was used for the determination of the stationary phase. Serial decimal dilutions of the cell suspension were prepared in 0.1% sterile saline peptone water up to the range of 8 log units. To determine the population of *Enterobacter aerogenes*, portions of 1 mL of the dilutions 10^{-6}, 10^{-7}, and 10^{-8} were deeply inoculated, and the standard Plate Count Agar (PCA) (Oxoid LTD, England) was then added. The PCA plates were incubated for 48 hours at 36 °C ± 1 °C. This procedure was performed in duplicate. For successive dilutions (2^{-1} to 2^{-9}), the 10^{-6} dilution was used to obtain a representative population. Each dilution used in the linearity test was performed in triplicate. In Figure 2, there is a

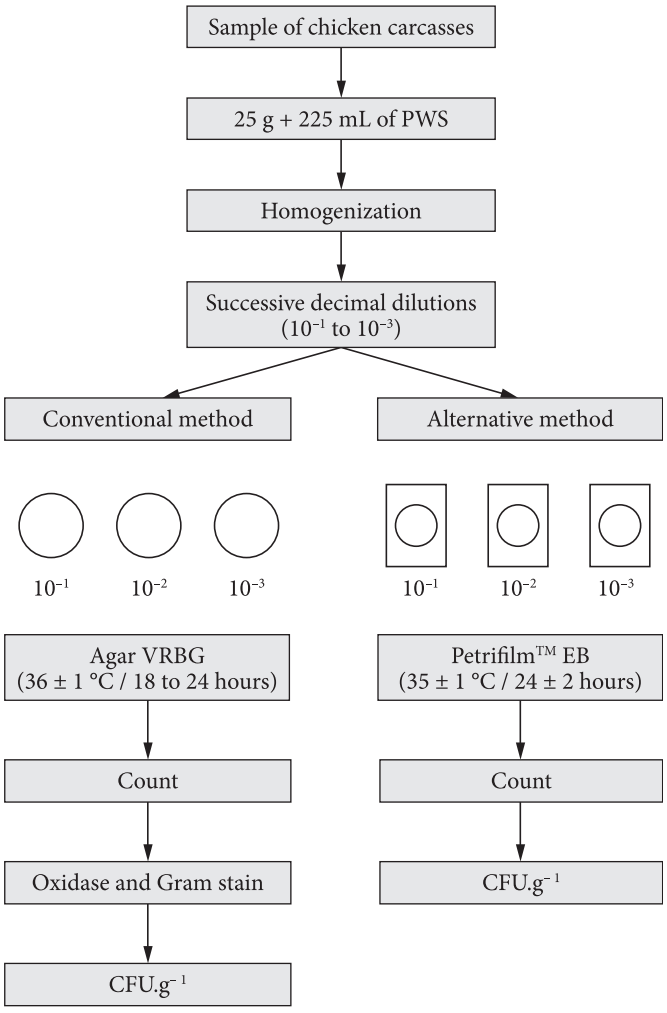

Figure 1. Schematic representation of the analysis by the method of *Enterobacteriaceae* Pour plate (BRASIL, 2003) and alternate by Petrifilm™ EB. PWS = peptone water saline 0.1%.

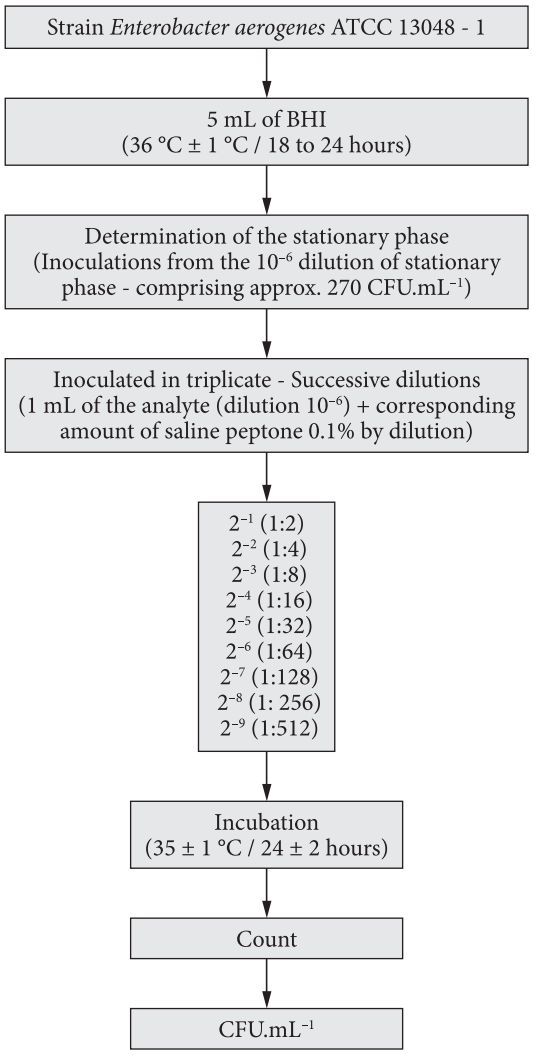

Figure 2. Schematic representation of procedure used to perform the linearity of the method Petrifilm™ EB.

schematic representation of the procedure used to perform the linearity test for the Petrifilm™ EB method.

Enterobacteriaceae population data obtained by both methods in each dilution were compared applying the Student's t-Test using the Microsoft Office Excel 2003 and BioEstat 5.0 for Windows software. The average of each dilution of each method was established to check for a statistically significant difference. With those results, using the BioEstat 5.0 software, the Student's t-Test was performed considering $p < 0.05$.

For the linearity test, applied to the Petrifilm™ EB method, the following formula was used (Equation 1):

$$G^2_{n-1} = 2\left[c_1 \ln\frac{c_1}{R_1} + c_2 \ln\frac{c_2}{R_2} + \ldots + c_n \ln\frac{c_n}{R_n} - \left(\sum c\right)\ln\left(\frac{\sum c}{\sum R}\right)\right] \quad (1)$$

where: G^2 corresponds to the dispersion index; c is the sum (Si) of the readings on 3 plates of the same level; and R corresponds to the relative volume at each level (RV) (INTERNATIONAL..., 2000).

The value of G obtained with the Chi-squared (χ^2) value tabulated with n-1 and 0.1% degrees of freedom was compared.

3 Results and discussion

From August to December 2009, 120 poultry samples were collected in different cities in the state of Rio Grande do Sul and submitted to *Enterobacteriaceae* enumeration to check whether there was statistical difference between the conventional and alternative methods. The linearity test was applied to the alternative method to confirm if the amount of analyte present in the sample corresponded to a linear or a proportional increase in results (INTERNATIONAL..., 2003).

When comparing the results of the three serial decimal dilutions used for both methods, a statistical difference ($p < 0.05$) was found (Figure 3). The lowest dilution obtained with the conventional method remained below the average or

could not be quantified sometimes. On the other hand, the Petrifilm™ EB method did not show a significant difference between the *Enterobacteriaceae* populations in the three different dilutions indicating better proportionality (Figure 3). Similar results were obtained by Silbernagel and Lindberg (2003). In general, alternative methods such as Petrifilm™ are considered practical and sensitive when compared to conventional methods (SILBERNAGEL; LINDBERG, 2002).

According to the literature, this is one of the first studies to address this kind of evaluation. The alternative Petrifilm™ EB method evidenced the following quantitative data in three different dilutions (Table 1).

Applying the formula for evaluating the linearity test, the value of the dispersion index found was (Equation 2):

$$G^2_{n-1} = 2\left[1566.036 - 1565.625\right] = 20.411 = 0.822. \quad (2)$$

The Petrifilm™ EB method was linear for the sample in a range between 1 and 256 colonies (2/3 = 0.6, i.e., 1 and 768/3 = 256). This can be observed in Table 1, considering the sum (Si) of the last dilution (2^{-9}) and the first one (2^{-1}) divided by the triplicates performed. This value should coincide with the relative volume used for the test.

Comparing the G value (0.822) with the Chi-square value (χ^2_{9-1} = 26.125 with n-1 and 0.1% degrees of freedom) the linearity of the results was proven (0.822 < 26.125). The values that remained below the theoretical value tabulated for n-1 and 0.1% degrees of freedom show that there was no dispersion of the values and the linearity of the results is acceptable.

The linearity of a method can be observed by correlating tests results and the analyte concentration. The correlation coefficient (R^2) is frequently used to indicate how much the straight line can be considered. The value of the coefficient $R^2 = 0.9992$ is close to 1; therefore the method can be considered bias free (Figure 4).

There are few studies found in the literature comparing the conventional and the Petrifilm™ EB method on poultry samples, but none of them compared the linearity of the alternative method. Silbernagel and Lindenberg (2003) compared the use of Petrifilm™ EB with the conventional method using samples of cheddar cheese, milk, flour, frozen prepared meals, nuts, and frozen broccoli. For most of the foods tested, there were no statistically significant differences between the data obtained by the Petrifilm™ EB method and the conventional VRBG method. The average score and the estimated accuracy of repeatability/reproducibility of the Petrifilm™ EB plates were quite similar.

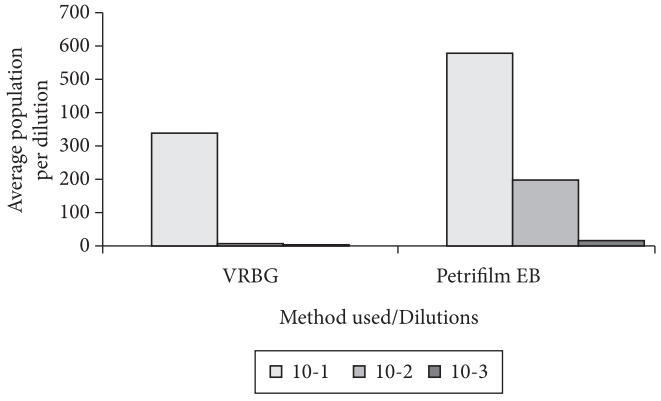

Figure 3. Comparison between means of dilutions used between VRBG and Petrifilm™ EB.

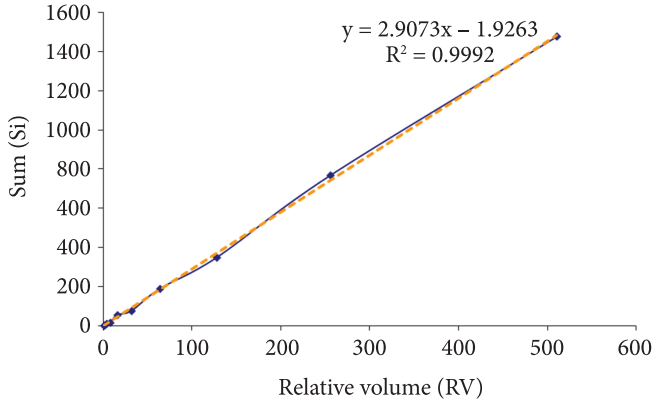

Figure 4. Linear correlation coefficient (R^2) and trend line for the quantification Petrifilm™ EB method.

Table 1. Quantitative data obtained in three dilutions for linearity tests of the Petrifilm™ EB method.

Dilution	Results Count (3 plates/dilution: X1, X2 and X3)							
	Plate 1 X1	Plate 2 X2	Plate 3 X3	Σ (Si)	Relative volume (RV)	Ratio (Si/RV)	Log N of Si/RV	Si × Log N
2^{-1} (1:2)	232	262	274	768	256	3.00	1.099	844.032
2^{-2} (1:4)	106	125	118	349	128	2.70	0.993	346.557
2^{-3} (1:8)	61	69	60	190	64	2.97	1.088	206.720
2^{-4} (1:16)	28	22	27	77	32	2.40	0.875	67.375
2^{-5} (1:32)	17	22	17	56	16	3.50	1.253	70.168
2^{-6} (1:64)	04	07	06	17	08	2.12	0.751	12.767
2^{-7} (1:128)	03	01	08	12	04	3.00	1.099	13.188
2^{-8} (1:256)	03	01	01	05	02	2.50	0.916	4.580
2^{-9} (1:512)	01	01	00	02	01	2.00	0.693	1.386
Total	-	-	-	1476	511	2.89	1.061	1566.036

When pure cultures were used, the Petrifilm™ EB method was as sensitive as the VRBG method and more selective than the conventional method. A total of 60 from 62 cultures of *Enterobacteriaceae* were recovered by both methods; however, Petrifilm™ EB inhibited the growth of a greater number of non-*Enterobacteriaceae* (SILBERNAGEL; LINDBERG, 2002).

In general, there are comparative Petrifilm™ studies in literature for the enumeration of indicator microorganisms such as mesophiles, staphylococci, coliforms, and *Escherichia coli* (BELOTI et al., 2003; SANT'ANA; AZEREDO, 2005; SANT'ANA; CONCEIÇÃO; AZEREDO, 2002; WATANABE et al., 2006).

Beloti et al. (2003) compared the use of Petrifilm™ EC and Petrifilm™ HS to the Most Probable Number (MPN) method to determine their efficiency in the enumeration of total coliforms and *Escherichia coli* in 145 water samples (76 freshwater samples and 69 water supply samples). For the fresh water samples, Petrifilm™ HS and Petrifilm™ EC showed good correlation with the MPN method. In chlorinated water (<20 colonies/MPN and negative),the agreement was low. For the enumeration of *E. coli*, Petrifilm™ EC showed good correlation with the MPN method. It can be said that Petrifilm™ EC and Petrifilm™ HS can be safely used for the enumeration of total coliforms and *E. coli* in water since the expected counts are higher than 20 CFU.100 mL^{-1}.

Sant'ana and Azeredo (2005) compared the use of Petrifilm™ RSA and the conventional methodology for the enumeration of coagulase-positive *Staphylococcus* in 62 samples of different foods. The authors observed that the Petrifilm™ RSA method differed significantly from the conventional method offering higher count averages. They also mentioned that the use of the alternative method for the enumeration of coagulase-positive *Staphylococcus* in foods is an alternative because of the short time (31 hours) to obtain quantitative results.

Watanabe et al. (2006) used Petrifilm™ AC to assess the level of water contamination in high-speed dental equipment. These researchers emphasized the practicality and the cost-benefits of the alternative method steps when compared to the steps of the conventional methods.

Traditionally, the enumeration of coliforms is used in food industries to evaluate hygienic and sanitary conditions. Detection and enumeration of Gram-negative fermenting microorganisms or *Enterobacteriaceae* family organisms in food industries has been more and more common. It is believed that the enumeration of a wider group of organisms, such as the *Enterobacteriaceae*, instead of a specific group, such as the coliforms, provides a deeper evaluation of the foodstuff (SILBERNAGEL; LINDBERG, 2003).

In Brazil, the Ministry of Agriculture, Livestock, and Supply (MAPA), according to the Normative Instruction Nº. 40 (BRASIL, 2005), has approved the use of Petrifilm™ EC as an official method to detect *Escherichia coli* on cattle carcass samples. The same Ministry has recently approved the use of Petrifilm™ AC for the enumeration of aerobic mesophiles and Petrifilm™ CC for thermotolerant coliforms in foods; however, for *Enterobacteriaceae* there is no information.

4 Conclusions

Through this study, it was observed that the two methods (VRBG and Petrifilm™ EB) showed a statistical difference ($p < 0.05$) when comparing the populations obtained in the three dilutions.

The Petrifilm™ EB quantification system was linear for the sample in a range between 1 and 256 colonies according to the linearity test.

Based on these results, it can be concluded that the alternative method showed greater consistency between the data obtained, gave results in a shorter time, and required no confirmatory biochemical tests.

Acknowledgements

The authors are extremely thankful to Professor Marli Luiza Brust for the final reading of the manuscript and to 3M Company for technical support.

References

ASSOCIATION OF OFFICIAL ANALYTICAL CHEMISTS – AOAC. Official Method: 2003.01. Enumeration of *Enterobacteriaceae* in Selected Foods. In: ANDREWS, W. H. (Ed.). **Microbiological Methods**. 18th ed. AOAC, 2006. cap. 17.3.10.

BELOTI, V. et al. Avaliação do Petrifilm™ EC e HS para enumeração de coliformes totais e *Escherichia coli* em água. **Brazilian Journal of Microbiology**, v. 34, n. 4, 2003.

BRASIL. Instrução Normativa nº 62, de 12 de dezembro de 2005. Métodos Analíticos Oficiais para Análises Microbiológicas para Controle de Produtos de Origem Animal e Água. **Diário Oficial da República Federativa do Brasil**, Brasília, DF, 16 dez. 2005. Seção 1, p. 70. Disponível em: <http://extranet.agricultura.gov.br/sislegis-consulta/consultarLegislacao.do?operacao=visualizar&id=15177>. Acesso em: 10 dez. 2009.

BRASIL. Instrução Normativa nº 62, de 26 de agosto de 2003. Métodos Analíticos Oficiais para Análises Microbiológicas para Controle de Produtos de Origem Animal e Água. **Diário Oficial da República Federativa do Brasil**, Brasília, DF, 18 set. 2003. Seção 1, p. 14. Disponível em: <http://extranet.agricultura.gov.br/sislegis-consulta/consultarLegislacao.do?operacao=visualizar&id=2851>. Acesso em: 23 jun. 2009.

FORSYTHE, S. J. **Microbiologia da Segurança Alimentar**. Porto Alegre: ARTMED, 2002. p. 1-378.

FRANCO B. D. G. M. **Métodos rápidos de análise microbiológica de alimentos**: estudo crítico e avaliação de novas metodologias. Tese (Livre Docência)-Universidade de São Paulo, São Paulo, 1994. Disponível em: <http://www.teses.usp.br/teses/disponiveis/livredocencia/9/tde-26052008-141940/pt-br.php>. Acesso em: 10 dez. 2009.

INTERNATIONAL ORGANIZATION FOR STANDARDIZATION - ISO. **ISO 16140**: Microbiology of food and animal feeding stuffs – Protocol for the validation of alternative method. ISO, 2003. p. 14-15;65.

INTERNATIONAL ORGANIZATION FOR STANDARDIZATION – ISO. **ISO/TR 13843**: Water quality - Guidance on validation of microbiological methods. ISO, 2000. p. 38-39. Technical Report.

JAY, J. M. **Microbiologia de Alimentos**. 6. ed. Porto Alegre: ARTMED, 2005. p. 1-712.

KORNACKI, J. L.; JOHNSON, J. L. Compendium of Methods For The Microbiological Examination Of Foods. In: DOWNES, F. P.; ITO, K. **American Public Health Association (APHA)**. 4th ed. Washington, 2001. cap. 8, p. 69-82.

SANT'ANA, A. S.; AZEREDO, D. R. P. Comparação entre o sistema Petrifilm RSA® e a metodologia convencional para a enumeração de estafilococos coagulase positiva em alimentos. **Ciência e Tecnologia de Alimentos**, v. 35, n. 3, 2005.

SANT'ANA, A. S.; CONCEIÇÃO, C.; AZEREDO, D. R. P. Comparação entre os métodos rápidos Simplate® TPC-CI e Petrifilm™ AC e os métodos convencionais de contagem em placas para a enumeração de aeróbios mesófilos em sorvetes. **Ciência e Tecnologia de Alimentos**, v. 22, n. 1, 2002.

SILBERNAGEL, K. M.; LINDBERG, K. G. 3M™ Petrifilm™ enterobacteriaceae count plate method for enumeration of enterobacteriaceae in selected foods: collaborative study. **Journal of AOAC International**, v. 86, n. 4, 2003.

SILBERNAGEL, K. M.; LINDBERG, K.G. Evaluation of the 3M Petrifilm Enterobacteriaceae Count Plate Method for the Enumeration of *Enterobacteriaceae* in Foods. **Journal of Food Protect**, v. 9, p. 1452-1456, 2002.

TRABULSI, L. R. **Microbiologia**. 4. ed. São Paulo: ATHENEU, 2005. p. 269-276.

WATANABE, E. et al. Diferentes métodos de avaliação do nível de contaminação microbiana da água de alta rotação. **Robrac**, v. 15, n. 40, 2006.