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ASSESSMENT OF THE EFFICIENCY OF SIMPLATE™ TOTAL PLATE COUNT COLOR INDICATOR (TPC CI) TO QUANTIFY MESOPHILIC AEROBIC MICROORGANISMS IN PASTEURIZED MILK

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ABSTRACT

The SimPlate™ TPC CI system is a rapid method to count mesophilic aerobic microorganisms (MAM) in foods, based on the use of resazurine to indicate bacterial growth. Its efficiency in pasteurized milk was evaluated using 142 pasteurized milk samples (38 type A, 43 type B and 61 type C) collected in Londrina, PR. The standard plating method, using Plate Count Agar (PCA) was used for comparison. The plates of both systems were incubated at 35ºC and read after 24h and 48h. The occurrence of false-positive and false-negative wells and the predominant microorganisms in them were also evaluated. The results were compared by simple correlation and mean variance analyses. The correlation (r) and mean variance values were 0.6811 and 0.7583 for the results obtained after 24h, respectively, and 0.9126 and 0.0842 for the results obtained after 48h, respectively. These results indicate that the performance of the system increases when the plates are incubated for 48h. When the three types of milk were evaluated separately, these values were 0.9285 and 0.0817 for type A milk, 0.9231 and 0.0466 for type B milk and 0.7209 and 0.1082 for type C milk. These results indicate that the better the quality of the milk the better the performance of SimPlate™ TPC CI. False-negative wells, found more frequently in samples with high MAM counts, were caused by Gram positive microorganisms, poorly detected by the SimPlate™ TPC CI system because they grew slowly and had low reduction capacity. The results indicated a higher efficiency of the SimPlate™ TPC CI system in the reading at 48h.

Key words: rapid methods, milk, SimPlate

INTRODUCTION

In the dairy industry, microbiological testing is important as milk is a highly perishable food. Milk may be a vehicle of pathogenic microorganisms, having caused various outbreaks of food poisoning (9,16).

The current methodology for microbiological testing of milk was developed several decades ago and is extremely troublesome and slow. It is based on conventional plating to count mesophilic aerobic microorganisms (MAM) (12) and enumeration of hygiene indicating microorganisms (17).

Several alternative methods have been developed to facilitate this microbiological control (10,13), and are called rapid methods. These methods are simpler, require little material and give results in less time. The greater practicality often justifies the use of these systems (7).

The SimPlate™ TPC CI system, developed by IDEXX Lab. Inc., USA, and currently distributed by BioControl Systems Inc., USA (6), aims to supply a quick and practical counting of microorganisms in foods. The SimPlate™ TPC CI method uses a 84 well plate where the food is sown with the culture medium and then incubated for 24h at 35ºC. The quantity of

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microorganisms is determined by the number of wells that show a positive result. This number corresponds to a Most Probable Number, as determined by the chart supplied by the manufacturer.

The SimPlate™ TPC CI system is a new version of the SimPlate™ TPC plates, which were based on the Multiple Enzyme Technology (15). The culture medium contained various substrates that when metabolized released a fluorescent substance (4). In pasteurized milk, the SimPlate™ TPC showed variations in the fluorescence intensity of the positive wells, causing doubt in the readings and low correlation with the standard MAM counting method (1).

SimPlate™ TPC CI, recently released in the market, quantifies MAM in foods using resazurine as a growth indicator substance. The multiplication of microorganisms alters the redox potential of the medium, resulting in a change of the initial blue color to pink, due to resorufin formation, or from yellow to colorless by forming dihydroresorufin. As the TPC CI medium is not based on microorganism enzymatic reaction, it does not suffer the interference of enzymes present in the foods (14).

This study was carried out to assess the efficiency of SimPlate™ TPC CI in MAM enumeration in pasteurized milk, compared to the standard plate count method, and to detect possible limiting factors for its use in the Brazilian dairy industry. Due to particularities of the Brazilian milk microorganisms, the capacity of the main microorganism groups to reduce the resazurin and produce color alteration in the SimPlate™ TPC CI wells was assessed.

MATERIALS AND METHODS

Sample collection and preparation

A total of 142 pasteurized milk samples (38 type A, 43 type B and 61 type C) were collected at several commercial establishments in Londrina, PR, Brazil, and transported under refrigeration to the Laboratory for Inspection of Products of Animal Origin of State University of Londrina (LIPOA UEL). Serial decimal dilutions of each sample in 0.85% saline were prepared.

Standard MAM plate count method

The dilutions were deep plated in Standard Plate Count Agar (PCA) following Brasil - Mara procedures (8). The readings were made after 24h and 48h of incubation at 35ºC. Results were expressed in CFU/mL (Colony Forming Units/mL).

SimPlate™ TPC CI (Biocontrol Systems, Inc., Bellevue, WA, USA)

The samples were processed following the manufacturer's instructions. The culture medium was dissolved in 100 ml of sterile distilled water and distributed in sterile test tubes (9.0 mL per tube). One milliliter of the sample or the dilution was added to each tube, and the mixture was transferred to the SimPlate™ TPC CI plate. The inverted plates were incubated at 35ºC and read at 24h and 48h. Wells showing any color change from the initial blue were considered positive. The Most Probable Number of Colony Forming Units (MPN of UFC) was determined using the chart supplied by the manufacturer.

Statistical analysis

The results, converted in $\log_{10}$ units, were submitted to simple correlation analysis, using Excell (Microsoft Office 2000) and Statistica programs. The results were analyzed separately according to the type of the milk (A, B or C) and jointly as well.

Detection of false-positive and false-negative wells in the SimPlate™ TPC CI plates

After reading, the content of twelve negative wells, six positive after 24h and six positive after 48h was aspirated with a micropipette and deep plated in PCA. Plates were incubated at 35ºC and read after 24h and 48h. The negative wells in the SimPlate™ TPC CI presenting growth in PCA were considered false negative wells. Wells positive in the SimPlate™ TPC CI showing no growth in PCA after 48h were considered false positive wells.

Characterization of microorganisms

The colonies grown on PCA plates originated from positive and negative SimPlate™ TPC CI wells were isolated and submitted to Gram staining. Among these, forty-one strains, representing all morphotypes, were tested for capability to reduce the TPC CI medium. For this, strains were suspended in 0.85% saline and submitted to decimal solutions (5). Ten microliters of each dilution were distributed in empty SimPlate™ plates, in order to obtain 3, 30, 300, 3,000 and 30,000 cells/well. Each well was filled with 50 µL of TPC CI medium. As control, 10 µL of the dilutions were also deep plated in PCA. Plates were incubated at 35ºC and read after 24h and 48h.

RESULTS AND DISCUSSION

Comparison between standard MAM in plate count method and the SimPlate™ TPC CI

When results obtained using the two methods were compared (Table 1) the performance of SimPlate™ TPC CI read after 48h was better than at 24h, regardless the type of milk analyzed. The correlations between the methods and their respective mean variances were 0.9126 and 0.0842 at 48h and 0.6811 and 0.7583 at 24h. Figs. 1 and 2 show the results dispersion.

The results also indicate that the milk quality influenced the performance of the SimPlate™ TPC CI system, that is, the higher the quality of milk analyzed, the better the performance of the method. The same phenomenon was previously observed by Beloti *et al*. (3), who evaluated the performance of Petrifilm™ AC in pasteurized milk and concluded that the milk quality influenced the method performance.

Table 1. Comparison of results given by SimPlate™ TPC CI and the standard method for enumeration of mesophilic aerobic microorganism (MAM) in pasteurized milk.

Systems	*r*	r^2	*p*	*n*	*a*	*b*	*Mean variance*
type A milk							
PCA x SimPlate™ TPC CI 24h.	0.717	0.514	0	16	0.469	0.997	0.382
PCA x SimPlate™ TPC CI 48h.	0.929	0.861	0	25	0.853	0.303	0.082
type B milk							
PCA x SimPlate™ TPC CI 24h.	0.688	0.473	0	29	0.972	-1.124	1.166
PCA x SimPlate™ TPC CI 48h.	0.923	0.852	0	34	0.912	0.364	0.047
type C milk							
PCA x SimPlate™ TPC CI 24h.	0.634	0.402	0	40	0.930	-0.583	0.613
PCA x SimPlate™ TPC CI 48h.	0.721	0.520	0	56	0.806	0.768	0.108
Total							
PCA x SimPlate™ TPC CI 24h.	0.681	0.464	0	85	0.747	0.077	0.758
PCA x SimPlate™ TPC CI 48h.	0.913	0.833	0	115	0.934	0.212	0.084

r: correlation; r^2: determination coefficient; p: level of significance; n: number of samples; a and b are linear regression parameters.

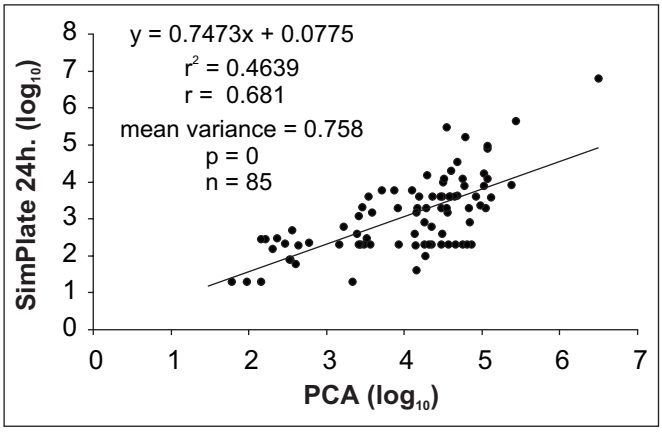

Figure 1. Correlation between counts of mesophilic aerobic microorganisms in pasteurized milk samples done in SimPlate™ TPC CI system and Plate Count Agar, after 24h of incubation.

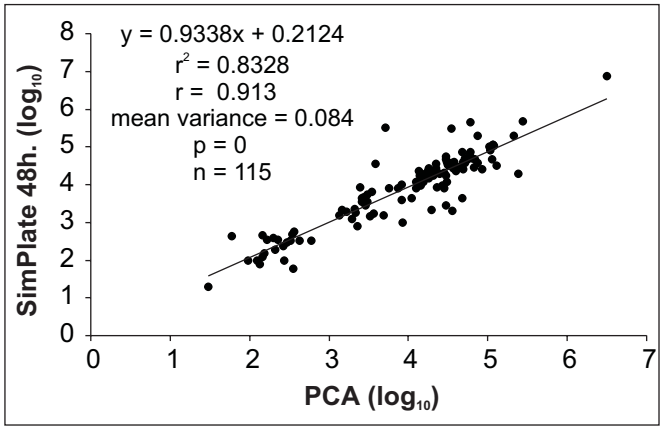

Figure 2. Correlation between counts of mesophilic aerobic microorganisms in pasteurized milk samples done in SimPlate™ TPC CI system and Plate Count Agar, after 48h of incubation.

False positive and false negative wells

From 1,188 negative wells plated in PCA, 70.71% presented no growth, indicating true negative results (Table 2). Among 517 wells considered positive at 24h or 48h, only one did not grow in PCA, indicating almost absence of false-positive results (Table 2).

The incidence of false-positive results varied according to milk type (Table 2). The lower frequency of false negative wells ocurred in type A milk (0.76%), followed by type B milk (31.62%) and by type C milk (33.70%). The results indicate the influence of the quality of milk on the SimPlate™ TPC CI performance. The better the quality, the lower is the incidence of false-negatives results.

The high percentage of false negative wells in pasteurized type B milk contrasts with the high correlation between the standard MAM count method and the SimPlate™ TPC CI (Table 1). This is probably explained by the industrial processing of this type of milk, in which samples go through a homogenization step. In this process, bacteria in groups are fragmented resulting in a better distribution throughout the milk, increasing the MAM count in PCA. When the milk is plated in SimPlate™ TPC CI, small volumes are transferred to the wells. These volumes are insufficient to give positive results in up to 48h incubation, but when the well contents are plated in PCA, the additional 48h incubation permits colony growth (13).

Table 2. Growth of well contents of SimPlate™ TPC CI in Plate Count Agar.

Observation	Plated wells	PCA result			
		negative		positive	
		n	%	n	%
type A milk					
negative wells	132	131	99.24	1	0.76
24h. positive wells	125	0	0.00	125	100.00
48h. positive wells	178	1	0.56	177	99.44
type B milk					
negative wells	427	292	68.38	135	31.62
24h. positive wells	28	0	0.00	28	100.00
48h. positive wells	48	0	0.00	48	100.00
type C milk					
negative wells	629	417	66.30	212	33.70
24h. positive wells	47	0	0.0	47	100.00
48h. positive wells	91	0	0.00	91	100.00
Total					
negative wells	1188	840	70.71	348	29.29
24h. positive wells	200	0	0.00	200	100.00
48h. positive wells	317	1	0.32	316	99.68

Characterization of microorganisms

Gram positive cocci, coccobacilli and bacilli predominated among the microorganisms retrieved from the SimPlate™ TPC CI wells (Table 3). In type A milk, the Gram positive coccobacilli was the predominant group.

Gram negative organisms were more frequent in wells presenting positive result at 24h (Table 4). Almost all microorganisms isolated from SimPlate™ TPC CI were able to reduce resazurin. Some needed 48h incubation to develop a positive response, indicating slow growth and low reducing capacity. Three Gram positive bacilli reduced resazurin even when the inoculum was as low as 13 cells/10µL.

Nine strains (3 Gram positive cocci, 3 Gram positive coccobacilli and 3 Gram positive bacilli) needed 48h to give a positive result, regardless the inoculum concen tration. Only one Gram positive bacillus strain did not present reduction capacity in 48h, even when the inoculun was as high as 298 cells/10µL.

Table 4. Frequency of microorganisms found in A, B and C type pasteurized milk isolated from negative and positive wells in the SimPlate™ TPC CI system after 24h and 48h incubation.

Gram classification	Microorganism Origin					
	negative wells		24 h. positive wells		48 h. positive wells	
	n	*%*	n	*%*	n	*%*
Cocci Gram +	119	*33.52*	65	*24.81*	126	*37.95*
Coccobacilli Gram +	136	*38.31*	82	*31.30*	89	*26.81*
Bacilli Gram +	74	*20.85*	61	*23.28*	95	*28.61*
Coccobacilli Gram -	4	*1.13*	32	*12.21*	10	*3.01*
Cocci Gram -	11	*3.10*	3	*1.15*	4	*1.20*
Bacilli Gram -	11	*3.10*	19	*7.25*	8	*2.41*
Total	355	*100.00*	262	*100.00*	332	*100.00*

This study showed that the majority of the microorganisms were able to reduce resazurine, but some needed a longer time or a heavier inoculum. These strains must be thermoduric microorganisms, and their low reducing capacity is well-known (2,11). The same microorganisms were responsible for the poor performance the Petrifilm™ AC for MAM enumeration in pasteurized milk (3). The low reduction capacity of these microorganisms are the cause of the poor performance of the rapid enumeration methods based on this principle.

CONCLUSIONS

In conclusion, the SimPlate™ TPC CI system is an interesting alternative to the standard plate count method, but for MAM enumeration in pasteurized milk it is recommended that the reading be made after 48h of incubation. The SimPlate™ TPC CI method is advantageous because is more practical in terms of labware and culture medium preaparation. It requires fewer dilutions so plating is more practical too.

Table 3. Frequency of microorganisms found in the SimPlate™ TPC CI system in A, B and C type pasteurized milk.

Gram classification	Total		Type A milk		Type B milk		Type C milk	
	n	*%*	n	*%*	n	*%*	n	*%*
Cocci Gram +	310	*32.67*	11	*15.71*	111	*36.51*	188	*32.70*
Coccobacilli Gram +	307	*32.35*	30	*42.86*	92	*30.26*	185	*32.17*
Bacilli Gram +	230	*24.24*	16	*22.86*	72	*23.68*	142	*24.70*
Coccobacilli Gram -	46	*4.85*	8	*11.46*	16	*5.26*	22	*3.83*
Bacilli Gram -	38	*4.00*	4	*5.71*	8	*2.96*	25	*4.35*
Cocci Gram -	18	*1.90*	1	*1.43*	4	*1.32*	13	*2.26*
Total	949	*100.00*	70	*100.00*	304	*100.00*	575	*100.00*

RESUMO

Avaliação da eficiência do SimPlate™ Total Plate Count Color Indicator (TPC CI) para enumeração de microrganismos aeróbios mesófilos em leite pasteurizado

O sistema SimPlate™ TPC CI é um método rápido para enumeração de microrganismos aeróbios mesófilos (MAM) em alimentos que utiliza a resazurina como substância indicadora de crescimento bacteriano. Para avaliar sua eficiência em leite pasteurizado, 142 amostras (38 de leite tipo A, 43 de leite tipo B e 61 de leite tipo C) foram colhidas em Londrina, PR, e analisadas pelo SimPlate™ TPC CI e pelo método de contagem em placas com ágar padrão de contagem (PCA). As placas de ambos os sistemas foram incubadas a 35ºC e as leituras realizadas em 24 e 48h. Também foi verificada a presença de resultados falso-positivos e falso-negativos bem como a microbiota láctea predominante nas cavidades do SimPlate™ TPC CI e a capacidade redutora dos diversos grupos de microrganismos. Os resultados foram comparados através de correlação simples e variância média. Considerando a leitura em 24h do SimPlate™ TPC CI, os resultados obtidos apresentaram uma correlação (*r*) de 0,6811 (var. média: 0,7583) com os resultados do método padrão; na leitura em 48h, a correlação encontrada entre os dois métodos foi de 0,9126 (var. média: 0,0842). Considerando os diferentes tipos de leite, as leituras em 48h do SimPlate™ TPC CI apresentaram as seguintes correlações com o método padrão: leite tipo A, *r*: 0,9285 (var. média: 0,0817); leite tipo B, *r*: 0,9231 (var. média: 0,0466); leite C, *r*: 0,7209 (var. média: 0,1082). Nas amostras com altas contagens de MAM, verificou-se uma maior freqüência de cavidades falso-negativas e uma grande participação de microrganismos Gram positivos, que são pobremente detectados pelo sistema SimPlate™ TPC CI por crescerem lentamente e possuírem baixa capacidade redutora. Os resultados indicaram um melhor eficiência do sistema SimPlate™ TPC CI na leitura em 48h, além da influência direta da qualidade do leite analisado, ou seja, quanto melhor a qualidade microbiológica do leite, melhor o desempenho do sistema. A alta correlação entre os métodos indica que o SimPlate™ TPC CI pode ser utilizado como uma alternativa viável ao método padrão de contagem de MAM em leite pasteurizado tipos A e B, desde que a leitura seja realizada em 48h.

Palavras-chave: métodos rápidos, leite, SimPlate

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