EVALUATION OF THE PROMICOL SYSTEM FOR STERILITY TESTING OF UHT DAIRY PRODUCTS COMPARING NEW AGE REAGENTS WITH CURRENT REAGENTS

ACTALIA Cecalait has evaluated the Promicol® System for the control of sterility of UHT products by comparison of new age reagents with current ones.

This method is used to detect microbial ATP (adenosine triphosphate) in a wide range of UHT and extended shelf-life (ESL) dairy products including flavoured milk, lactose reduced milks, desserts, baby milks and creams. After incubation for 2 or 3 days at 30°C, just after their production, milk products are analysed by Promicol method for evaluation of the growth of bacteria, by extraction and detection of their ATP.

The Promicol® System includes a microbial ATP detection kit specific for UHT and ESL dairy products, the PromiLite M4 luminometer and the Proscreen Software.

MATERIAL AND METHODS

In this study, Promicol® method using current reagents (kit 1) and new age reagents (kit 2) has been compared with official method for detecting non-sterile dairy products.

5 types of products were tested:

– UHT whole milk in brick of 1 l;
– UHT semi-skimmed milk in brick of 1 l;
– UHT chocolate milk in brick or glass bottles of 20 cl;
– UHT cream in brick or plastic bottles of 20 cl;
– Vanilla dessert in plastic cups of 115 g: for these products stored between 0 to 6°C, the criterion of the official method is not applicable.

Official method

The official method is described in the EU directive 94/71(1994) modifying the directive 92/46 (1992).
After incubation of products for 15 days at 30 ± 2°C, microorganisms at 30°C are enumerated by inoculation of 0.1 ml in Plate Count Agar with skimmed milk (mPCA) according to ISO 4833-1.
The criterion is ≤ 10 CFU for 0.1ml of milk corresponding to < 100 CFU/ml.
Remark: this criterion is not applicable for products stored usually at refrigerated temperature (e.g. dairy dessert)

In this study the enumeration was performed in 2 plates inoculated by 0.1 ml.
For dessert and cream, we obtained very opaque plates, difficult to read, so we inoculated the sample in 1 plate with 0.1 ml of product and in 1 plate with 0.1 ml of a decimal dilution.

Promicol® method

Promicol® test is performed after incubation of products for 2 days at 30 ± 2°C according to the procedure described in the supplier kit insert.

A microplate well is inoculated with 50 µl of product with an ATP free tip. For thick products, as creams and desserts, a widebore tip is used. After inoculation, the microplate containing 96 wells is put into the Promilite Luminometer, which, linked with the Proscreen Software V4.000, analyses automatically the samples. Some controls are performed for each microplate in the first wells.

Each sample was analysed with 4 repetitions for both reagents to determine the repeatability coefficient of variation expressed in percentage.

The results are expressed in Relative Light Units (RLU), with 3 types of interpretation according to table 1.

Table 1: Interpretation of results

REPLY	STERILITY OF PRODUCT	RLU VALUE
Pass	Sterile product	RLU < 2 x blank RLU
Retest	Product to be retested after additional incubation	2 x blank RLU< RLU < 3 x blank RLU
Fail	Non-sterile product	RLU > 3 x blank RLU

PROTOCOL

All samples were homogenized by hand (about 25 times), before being pipetted under aseptic conditions directly in the bottle or brick, or by a syringe in case of additional incubation. In this case the hole made by the syringe was closed by an adhesive sterile film.

Determination of blank values

20 samples of each type of products were analysed to determine the blank value used for the interpretation of Promicol® results (see table 1):

- By Promicol® method after **2 days** of pre-incubation at 30 ± 2°C, with 4 repetitions by new age and current reagents;
- By the official method after **2 and 15 days** of pre-incubation at 30 ± 2°C.

The blank values were then obtained by calculating the average of the 80 results.
For some types of products various brands were analysed, in this case the average value was calculated only if homogeneous values were obtained; otherwise a blank value was calculated for each brand.

Contaminated samples

Contaminations

5 strains were used for contamination, one strain for each type of product, and are described in table 2.

Table 2: Strains used for contamination

DAIRY UHT/ ESL PRODUCT	UHT whole Milk	UHT skimmed milk	UHT chocolate milk	UHT cream	ESL Vanilla dessert
Strain	*Bacillus cereus*	*Salmonella* Typhimurium	*Listeria mono-cytogenes* 1/2a	*Escherichia coli*	*Candida parapsilosis*
Internal number	14001	98002	00002	99008	14065
Origin	Food product	Dairy product	Raw milk	*Raw milk*	UHT milk

Each strain was cultivated in BHI broth 18 h at the optimal temperature. A volume of the diluted broth was used to obtain a contamination of 5-10-15 CFU per brick, bottle or cup**.** The contamination was repeated 2 times, giving 6 samples contaminated by 1 strain per product.

The samples were contaminated under aseptic conditions, with a volume of the diluted broth inoculated with a syringe through the aluminium cover, or directly through the brick. The hole made by the syringe was closed by an adhesive sterile film. The level of the contamination was verified by inoculating 1 ml in 5 PCA plates, at the same dilution as the contamination.

Analyses

6 samples of each type of products were analysed

- By Promicol® method after **2 and 3 days** of pre-incubation at 30 ± 2°C, with 4 repetitions by new age and current reagents;
- By the official method after **2, 3 and 15 days** of pre-incubation at 30 ± 2°C.

RESULTS

Determination of blank values

20 samples of each type of products were analysed 4 times by both kits to determine the blank value (average of 80 analyses). The coefficients of variation (CV) for 4 repetitions were calculated in percentage, and then the average of these values.
Results are summarised in table 3.

Table 3: Data on negative samples

DAIRY UHT/ ESL PRODUCT	20 UHT whole milk (3 brands)	20 UHT skimmed milk (4 brands)	20 UHT chocolate milk (3 brands)	UHT cream 2 brands		20 vanilla desserts (1 brand)
				20 (brand 1)	6 (brand 2)	
KIT1 Day 2 Mean in RLU	10	9	7	17	91	18
KIT2 Day 2 Mean in RLU	6	7	6	24	76	17
Total flora Day 2	neg	neg	neg	neg	neg	neg
Day 15	2 pos(1)	neg	neg	neg	neg	10 pos(2)

(1) *This contamination may be due to a cross contamination in the water bath and not to the sample itself.*

(2) *11 to 30 colonies were enumerated per plate, but these samples can be considered as blanks: official criterion (≤ 10 in 0.1 ml) is not applicable for these products stored in refrigerator and the number of colonies was very low.*

The blanks values were low (6 to 24 RLU) except for the second cream (76 and 91 RLU).
CV values were high (between 25% and 58%), this can be explained by the low values in RLU.

Contaminated samples

Artificially contaminated samples

Each couple product/strain was contaminated 2 times at 5, 10 and 15 CFU per packing unit, so 6 samples per product were analysed by both kits 4 times (24 results).

The real values of contamination were calculated from the enumeration in the 5 mPCA plates

Results are presented in the table 4.

Table 4: Contaminated samples data

DAIRY UHT/ ESL PRODUCT	6 UHT whole milk	6 UHT skimmed milk	6 UHT chocolate milk	6 UHT cream (Brand 2)	6 ESL vanilla desserts
Real CFU/ Bottle, brick or cup	7-14-21 *B. cereus*	5-10-15 *Salmonella*	3-6-10 *L. mono*	8-15-23 *E. coli*	7-14-20 *Candida parapsilosis*
KIT1 (n = 24) **RLU Day 2** **RLU Day 3**	 pos pos	 pos pos	 pos pos	 pos 21 pos/2 retest/1 neg	 20 pos/2 retest/2 neg pos
KIT2 (n = 24) **RLU Day 2** **RLU Day 3**	 pos pos	 pos pos	 pos pos	 23 pos/1 neg 20 pos/3 retest/1 neg	 18 pos/6 retest pos
Total flora (n = 6) **Day 2** **Day 15**	 pos pos	 pos pos	 pos pos	 pos pos	 pos pos

Mean values were high (between 200 and 9800 RLU), and gave always positive results except 3 negative for creams and 2 for desserts. We can suppose that when a retest is needed after a supplementary incubation (e.g. 1 day) the result will become positive.
We obtained low CV values (about 5%) for whole milk and chocolate milk, but higher CV values (between 26% and 56%) for:

- skimmed milks: may be due by spoilage of milk, that caused the heterogeneity of contamination;
- creams and desserts: due to their viscosity

Naturally contaminated samples

During examination of the blank samples for cream (brand 1) after 2 days at 30°C, 6 of them showed very high results with the Promicol® method, while no colonies were enumerated in mPCA after 2 days and 15 days at 30°C (see table 5). A contamination was observed only in surface of blood agar plates incubated at 30°C or 37°C. These results show that Promicol® method was more sensitive to detect this type of contamination than the official method.
The strain isolated from these samples was identified by Maldi-Tof method as *Bacillus sporothermodurans*, a spore forming bacteria which is known to be mesophilic in vegetative form (it can grow at 30°C) and very thermoresistant in spore form.

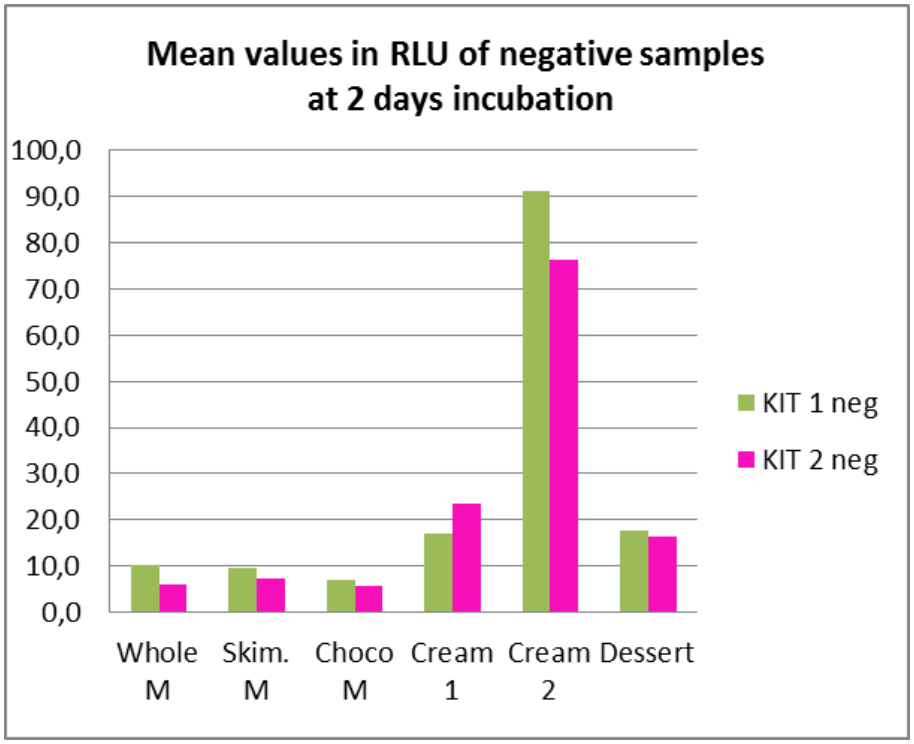
Table 5: Naturally contaminated samples data in cream (brand 1)

UHT cream (Brand 1)	20 blanks (blank value)	6 positives samples
KIT1 Day 2 **RLU**	mean = 17	270 - 4400
KIT2 Day 2 **RLU**	mean = 24	180 - 3000
Total flora **Day 2** **Day 15**	 Neg Neg	 Neg Neg*

***** *no growth in PCA at 30°C, but culture at surface of blood agar plates (0.1 ml at dilution -1) at 30°C and 37°C*

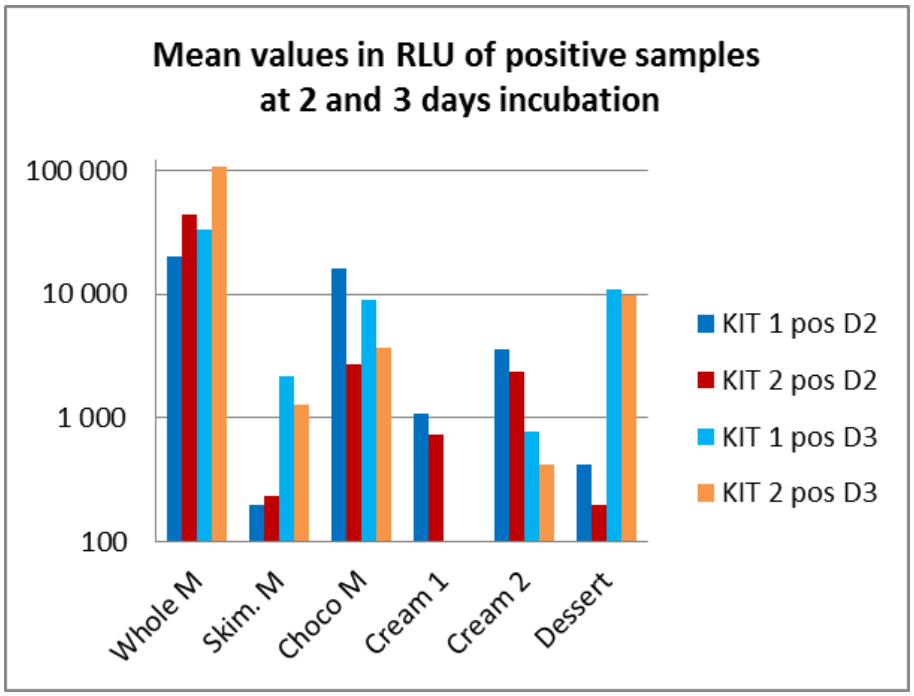
Comparison between kits

Figure 1: Comparison between kits for blank samples in mean RLU



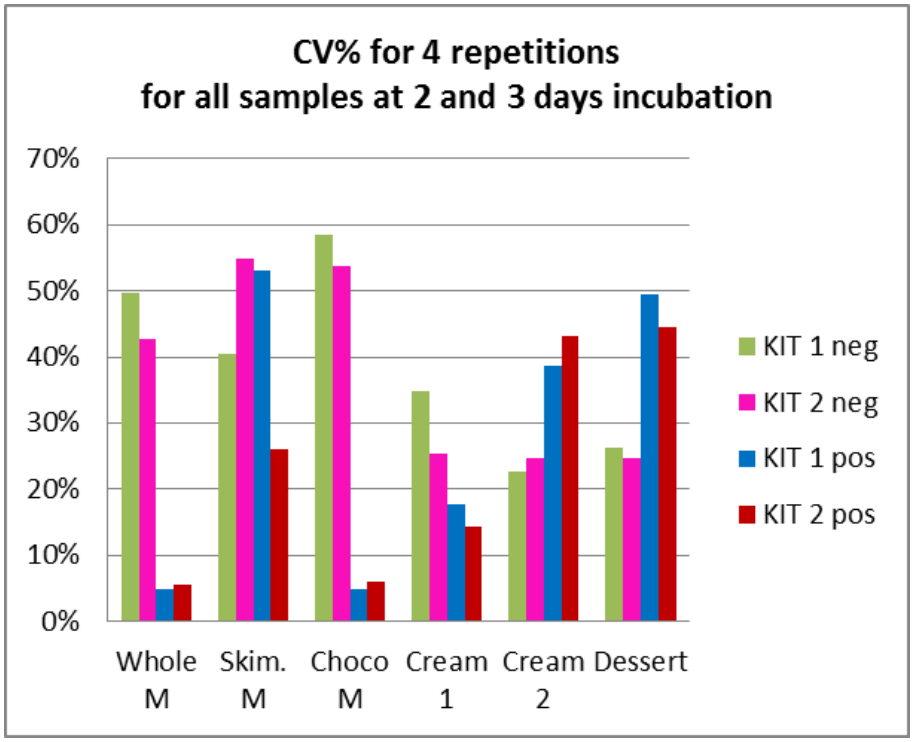
For the blank samples the RLU values are equivalent between the 2 kits and a little lower for the new one except for the cream 1.

Figure 2: Comparison between kits for the positive samples in mean RLU (in semi logarithmic scale)



For the positive samples the RLU values are equivalent between the 2 kits and often lower for the new one.

Figure 3: Comparison between kits for all samples in CV% for 4 repetitions



For all samples the CV% for 4 repetitions are equivalent between the 2 kits and in general a little lower for the new kit, and for the positive skimmed milk samples it is significantly lower.
For positive samples the high viscosity of dessert and cream can explain their high values of CV%. For the skimmed milk, it was rotten by the high level of contamination, involving the heterogeneity of the matrix.

CONCLUSION

Comparison between the current reagents (kit 1) and the new age reagents (kit 2):

- RLU values at 2 days of incubation for negative or positive samples are equivalent for the 2 kits, or sometimes lower for the new one;
- The coefficient of variation on 4 repetitions calculated in %, for blank or positive samples results are equivalent or lower for the new kit;
- For the 30 artificially contaminated samples after 2 and 3 days of pre-incubation at 30°C, the number of false negative results was equivalent on 240 analyses (3 negative for kit 1 c/ 2 negative for kit 2);
- The 6 naturally contaminated samples were well detected by both kits.

Performance of the Promicol® method for sterility testing in UHT or ESL products:

- Promicol® method is very easy to implement, as addition of reagents, optical reading and interpretation of results are automatic.
- Promicol® is a rapid method that needs a 2 days pre-incubation at 30°C and 20 min of analysis, versus 15 days of pre-incubation for the reference method followed by 3 days of incubation of PCA plates.
- The performance of the Promicol® method is equivalent or more sensitive than the official method in PCA, as showed in this study for the naturally contaminated samples of cream. The official method needs sometimes (e.g. for cream and dessert) a decimal dilution and the results in PCA are sometimes difficult to read in particular when cultures are overgrowing.
- Promicol® is relevant for all types of UHT and ESL products, while resazurin test, which is commonly used in dairy industry, cannot be used for coloured flavoured milk and certain types of supplemented milks.
- Even thick/viscous samples can be analysed with the Promicol® method, whereas usage of wide bore tips and thoroughly shaking before pipetting are recommended to ensure homogenous sampling