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Rapid Detection of Psychrotrophic Bacteria In Manufacturing Grade Raw Milks

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ABSTRACT

Methods to rapidly assess the bacteriological quality of raw milk were investigated. Whereas direct microscopic count, modified psychrotrophic plate count, and direct epifluorescent filter technique (DEFT) did not correlate well with initial psychrotrophic bacterial count of raw milk, improvements were obtained after preincubation of the milk samples. The best preincubation conditions were identified as 30°C for 6 h, 21°C for 10 h, 13°C for 15 h, 13°C for 20 h, or 7°C for 37 h.

The "square root" equation was applied to the data, and a model was produced for predicting growth of the native microflora of raw milk. Using this equation, a DEFT count after preincubation of the milk at 21°C for 10 h could accurately predict the initial psychrotroph count and the count after storage of the milk at 6°C for 48 h.

Freshly produced raw milk usually contains gram-positive bacteria, derived principally from the udder, together with varying numbers of gram-negative psychrotrophic bacteria from extraneous sources such as water and equipment contact surfaces. The numbers of the latter may vary depending upon hygienic and sanitary practices, quality of rinse water, and, to some extent, ambient seasonal temperature. Therefore, even milk produced under good hygienic conditions may contain a few gram-negative psychrotrophic bacteria. Though these constitute a minor part of the milk microflora immediately after milking (<10%), their numbers rapidly increase due to their ability to grow at refrigeration temperatures encountered in bulk tank milk (3-5°C). By the time the milk reaches the processing plant, these organisms have become the dominant flora. At this point, even raw milk of good quality may have psychrotrophic bacterial counts in the range of 1×10^3 to 1×10^4 CFU/ml (*20,33*). Though psychrotrophic bacteria have been shown to cause keeping quality problems (sensory defects) in fluid milk at levels of 1×10^4 CFU/ml (*16*), generally higher levels ($>1 \times 10^6$ CFU/ml) have been associated with yield losses and off-flavors of aged Cheddar cheese (*1,2,12,13,17*). Therefore, the cheese manufacturer needs to know the initial psychrotrophic bacterial count of the incoming raw milk in order to determine the maximum length of refrigerated storage not prejudicial to cheese yield or quality.

Standard methods for determination of psychrotrophic bacterial count require incubation of plates at 7°C for 10 d. This is not suitable for routine quality control. Oliveria and Parmelee (*23*) developed a more rapid modified psychrotrophic bacterial count that required incubation of plates for 25 h at 21°C. There was a good correlation between counts obtained by this method and the standard psychrotroph count procedure (*3,4,9,23,33*). In order to obtain results even more quickly, Zall et al. (*34*) used a preincubation step of 5 h at 30°C coupled with a direct microscopic clump counting method to enumerate psychrotrophs in milk. They reported a high correlation between their method and that of Oliveria and Parmelee (*23*). Because a preincubation temperature of 30°C does not exclude the growth of mesophiles, preincubation at 21°C or 13°C may be more suitable for selection of psychrotrophic growth (*3,4,9*). Lengthy plate count incubation procedures that limit the usefulness of the techniques for routine screening of raw milks are required after the preincubation steps.

The direct epifluorescent filter technique (DEFT) is a rapid method for enumeration of bacteria that can differentiate between viable and nonviable cells and correlates well with the standard plate count even at levels of 1×10^3 to 1×10^4 CFU/ml (*7,25,28,29*). When coupled with psychrotrophic preincubation conditions, DEFT may serve as a suitable rapid index of psychrotrophic bacterial count.

MATERIALS AND METHODS

Milk samples

Raw milk samples from 25 producers supplying two cheese plants and 24 producers supplying one plant were obtained in each of four seasons. This sampling plan should have resulted in 100 samples from each of two plants and 96 from one plant for a total of 296 samples. However, during the course of this study some of the producers (fall and winter) were no longer available, and these were not replaced. Thus, only data from 283 samples were used for statistical analysis.

Sampling was carried out all through the year. Samples of milk (120 ml) were removed aseptically from farm bulk tanks into sterile WhirlPak bags by milk haulers. The samples were transported to the factories in ice chests and were picked up for

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removal to the laboratory within 3 h of delivery. The temperature on receipt in the laboratory was between 1.7 and 6.7°C.

Preincubation conditions

Before incubation, the milk samples were subjected to a sonication treatment (20 min; Bronson 3200 Model) to disrupt clumps of bacteria (*31*). Disaggregation of bacterial clumps by agitation (*21*), shaking, or by an ultra-turrax for 30 s at 20,000 rpm (*7*) improved the accuracy of DEFT and its correlation with plate count. The treated milk was divided into five aliquots. Three aliquots were placed in a water bath at 30°C to equilibriate samples to the desired incubation temperature of 13, 21, or 30°C. After the samples reached the desired temperature, they were transferred to the respective incubator. Temperature was monitored with a glass thermometer placed in the same volume of milk in a test tube as the milks being tempered in the water bath. The 7°C aliquots did not require pretempering because they were already in the range of 2-6°C and were expected to reach 7°C within a short time. The fifth aliquot of milk was retained for bacteriological analysis. Preincubation conditions used are set out in Table 1.

TABLE 1. *Preincubation conditions used in the study.*

Temperature (°C)	Preincubation time (h)
7	32
	37
	42
13	10
	15
	20
21	10
	17
	24
30	3
	6

Bacteriological analysis

Plate counting. Plate count agar plates were inoculated using a Spiral Plater (Spiral Systems, Inc., Bethesda, MD) (*14*). Standard incubation temperatures were used for standard plate count (SPC) (*19*) and psychrotrophic bacterial count (PBC) (*8*). In addition, the modified psychrotrophic bacterial count (mPBC) was obtained after incubation of plates at 21°C for 25 h (*23*).

Direct microscopic count (DMC). DMC was performed as described by Zall et al. (*33*) on a limited number of samples (113). Analysis of 74 samples from spring (all three plants) and 39 from summer (two plants) revealed poor correlations initially and after preincubation of samples at 13 or 21°C. Therefore, no further sampling was done for DMC.

Direct epifluorescent filter technique (DEFT). The method of Rodrigues and Kroll (*28*) was used.

Statistical analysis

Microbial counts after $\log_{10}$ transformation were analyzed with SAS software (SAS User's Guide, SAS Institute, Inc., Cary, NC) on the mainframe computer at the University of Minnesota.

RESULTS AND DISCUSSION

Comparison of count procedures

Average microbial counts determined by the various procedures are shown in Table 2. The highest counts were obtained by DMC and DEFT. PBC and mPBC were very similar. SPC were slightly higher than PBC and mPBC but similar to those obtained by DEFT. DMC measured both viable and dead bacterial cells as the DMC was 1.5-log cycles higher than the SPC. On the other hand, the average count obtained using DEFT was only 0.3-log cycles higher than SPC, indicating that the former was probably detecting only viable cells that can be distinguished by the color of fluorescence (*25*). All counts were higher in the winter than in the three other seasons (Table 3), and this reflects changing husbandry practices. The overall level of PBC, mPBC, and SPC was nearly the same for producers supplying the three plants (Table 4). However, DEFT counts were higher with milks from creamery B. DMC was again the highest obtained, and the highest of these was observed for milks from creamery C. Since the average DMC was 1 log higher than the DEFT or SPC, it would appear that there were some dead bacteria present in these milks from each plant (Table 4). These data also indicated very high initial PBC of >1 x 10^7 CFU/ml in some milks, perhaps due to higher storage temperatures and growth of psychrotrophs (temperature of incoming milks were in the range 1.7 - 6.7° C).

TABLE 2. *Average bacterial counts obtained by various counting procedures in manufacturing grade producer milks.*

Counting procedure	No. of samples	Log average	Standard deviation in log units	Average count*
Psychrotrophic bacterial count (7°C/ 10 d)	283	4.43	1.31	2.7 x 10^4
Direct microscopic count	113	6.50	1.11	3.2 x 10^6
Direct epifluorescent filter technique	238	5.30	1.06	2.0 x 10^6
Modified psychrotrophic bacterial count (21°C/25 h)	283	4.50	1.25	3.2 x 10^4
Standard plate count	283	5.02	1.12	1.1 x 10^5

*Antilog of the geometric average.

TABLE 3. *Variation in plate counts of manufacturing grade producer milks with season.*

Counting procedure	Average count (CFU/ml)* Spring	Summer	Fall	Winter
No. of Samples	74	74	69	66
Psychrotrophic bacterial count (7°C/10 d)	2.1×10^4	1.3×10^4	1.6×10^4	1.3×10^5
Modified psychrotrophic bacteria count (21°C/25 h)	1.9×10^4	1.1×10^4	3.1×10^4	1.7×10^5
Standard plate count**	4.8×10^4	6.0×10^4	7.7×10^4	6.0×10^5

*Antilog of the geometric average.
**Standard deviations in log units were in the same order of magnitude as in Table 1 except in spring they were 0.25-0.5-log units higher.

TABLE 4. *Variation in counts obtained by different procedures for manufacturing grade producer raw milks supplied to three different cheese plants.*

Counting procedure	Average Creamery count*/ml A	B	C
	No. of samples 98	91	94
Psychrotrophic bacterial count (7°C/10 d)	3.0×10^4	3.5×10^4	1.5×10^4
Modified psychrotrophic bacterial count (21°C/25 h)	4.3×10^4	4.6×10^4	1.7×10^4
Standard plate count	1.2×10^5	8.2×10^4	8.2×10^4
Direct epifluorescent filter technique	1.4×10^5	6.9×10^5	1.0×10^5
Direct microscopic count**	1.8×10^6	1.2×10^6	7.6×10^6

*Antilog of the geometric average.
**Based on 45, 24, and 44 samples from A, B, and C, respectively.

The average counts of mPBC and SPC of these producer milks were about 10-fold higher than those reported for individual producer milks from New York (*33*) but were similar to counts observed on commingled milk from the same source (*33*). There was reasonable agreement (r=0.8) between PBC and mPBC, but this was not as good as has been previously reported (*4,9,33*). Both DMC and DEFT showed poor correlation with the initial PBC (r=0.39 and 0.21, respectively). This fact results from the inability of these procedures to differentiate between mesophilic and psychrotrophic bacteria. The presence of large numbers of dead cells that were counted by DMC would contribute to the disparity in counts. To a certain extent this also may be true of the DEFT as some gram-positive bacteria, especially streptococci, stain orange with acridine orange regardless of their viability (*15*).

Preincubation procedures

The correlation of PBC with mPBC increased significantly after the milk was subjected to certain preincubation procedures (Table 5). Preincubation conditions of 30°C/6 h, 21°C/10 h, 13°C/15 h, 13°C/20 h, and 7°C/37 h were particularly effective. This was in agreement with previous reports (*4*). The correlation decreased significantly after 24 h at 21°C, suggesting growth of mesophiles. The correlations of PBC with DEFT and DMC also increased after preincubation (Table 5). However, the correlation coefficient was never higher than 0.73 due to inherent differences in the techniques and lack of distinction between (a) psychrotrophs and mesophiles and (b) viable and dead cells by the microscopic methods. The latter would be most relevant to DMC which, indeed, showed higher average counts; in some cases being over 1×10^7 CFU/ml. Higher average DEFT counts than PBC, mPBC, or SPC were seen with milks from creamery B. These milks had higher levels of streptococci than samples from the other creameries, and this may have been a contributing factor to the raised DEFT counts (*15*).

The correlations obtained during comparisons of the various counting procedures were affected by both season and origin of the milk (Table 6). This may simply reflect differences in the microflora of milk from the different sources and throughout the year. Correlations between PBC and DEFT, in the majority of cases, improved after a period of preincubation at 21°C for 10 h providing the best conditions. The relationship between PBC and mPBC also became more significant after preincubation, except in the case of milks sampled in the winter when little change was observed. Preincubation for 37 h at 7°C provided the best conditions to optimize the relationship between PBC and mPBC. DMC was unsuitable as a rapid indicator of count even after preincubation. Even though the correlation of PBC with mPBC and DEFT increased significantly after pre-incubation at 21, 13, and 7°C, the preincubated mPBC showed poor correlation with the initial PBC (Table 7). This was in agreement with the findings of Bishop and Juan (*4*). This might relate to differences in the initial ratios of component microflora, their relative growth rates at preincubation temperatures, and their ability to form colonies on plates within 25 h at 21°C. Evidence (data not shown) indicated that the relationship between PBC and mPBC and between PBC and DEFT were dependent upon the source of the milk. The correlations for these comparisons varied when milks from different plants were studied. Thus, in practice, different preincubation conditions might be needed at different processing sites.

Prediction of psychrotrophic bacterial count of milk

The establishment of a model that describes the relationship between temperature and growth rate of psychrotrophs and mesophiles would aid identification of a critical temperature zone that may be suitable for detection of psychrotrophs. The generation times at 30, 21, 13, and 7°C were calculated from the present data and used in the "square root" model of Ratkowsky et al. (*27*). The extrapolated T_0 value (notional minimum growth temperature) ranged from 210 to 280°K for the native microflora of the

TABLE 5. *Linear correlation coefficients between the psychrotrophic bacteria count (7°C/10 d) and modified psychrotrophic bacteria count (21°C/25 h), direct microscopic, and direct epifluorescent microscopic counts of manufacturing grade producer milks* under varying conditions of preincubation.*

Correlation between	Initial	After preincubation at temperatures and times indicated										
		30°C		21°C			13°C			7°C		
		3 h	6 h	10 h	17 h	24 h	10 h	15 h	20 h	32 h	37 h	42 h
PBC:MPBC (283 samples)	0.79	0.82	0.86	0.88	0.83	0.53	0.80	0.89	0.79	0.79	0.95	0.95
PBC:DEFT (283 samples)	0.39**	0.41	0.60	0.60	0.60	0.18	0.41	0.60	0.54	0.61	0.68	0.73
PBC:DMC (113 samples)	0.21	0.35	0.13	0.63	0.68	0.21	0.45	0.31	0.44	0.48	0.68	0.53

*Based on 283 samples.
**r value of PBC:DEFT initial was based on 238 samples and all others after preincubation were based on 283 samples.

TABLE 6. *Influence of season and region of supply of manufacturing grade producer milks on correlations among bacterial counts.*

Source	No.	Preincubation conditions									
		None		30°C-6 h		21°C-10 h		13°C-15 h		7°C-37 h	
		PBC vs. DEFT	PBC vs. MPBC	PBC vs. DEFT	PBC vs. MPBC	PBC vs. DEFT	PBC vs. MPBC	PBC vs. DEFT	PBC vs. MPBC	PBC vs. DEFT	PBC vs. MPBC
All samples	283	0.39*	0.79	0.41	0.86	0.61	0.88	0.60	0.89	0.68	0.95
Creamery B	91	0.10	0.83	0.38	0.91	0.59	0.91	0.59	0.87	0.60	0.97
Creamery A	98	0.56	0.39	0.39	0.88	0.75	0.92	0.66	0.90	0.74	0.94
Creamery C	94	0.51	0.72	0.36	0.77	0.70	0.82	0.70	0.88	0.70	0.93
Summer	74	0.29	0.77	0.55	0.85	0.83	0.96	0.72	0.83	0.75	0.96
Spring	74	0.23	0.70	0.24	0.83	0.64	0.85	0.41	0.89	0.51	0.88
Fall	69	0.50	0.75	0.30	0.91	0.49	0.71	0.67	0.92	0.60	0.95
Winter	66	0.64	0.93	0.60	0.83	0.70	0.89	0.66	0.95	0.68	0.95

*Based on 238 samples.

milks studied (Fig. 1). The values below 250°K may reflect the presence of truly psychrophilic bacteria in the milk (*5,21*) and/or inaccuracies in calculation of generation times due to high initial bacterial populations in some milks. Therefore, data for only the milks (84 samples) that had initial PBC in the range of 1×10^2 to 1×10^6 CFU/ml and extrapolated T_o values of 250 to 280°K were used to develop the model (Fig. 2). The average T_o value from the regression equation was 265°K. This was similar to values reported previously for the growth of the native microflora in raw and pasteurized milks (*5,10,11*). Because the model was based on many milks expected to contain several different groups of psychrotrophs, generation times derived from this regression may not apply to a single psychrotroph but would be expected to provide an average that would be applicable in practical situations.

Formulation of a more expansive "square root" model based on data from a number of sources (*18,21,30*) as well as the present study, resulted in a T_o value of 265.4°K (Fig. 3). In agreement with previous observations (*11,26*), results also indicated that the T_o values and generation times of psychrotrophs varied considerably (Table 8) especially at refrigeration temperatures (4-7°C). At 10°C and above, their generation times were similar and of the same order as found by Griffiths and Phillips (*11*). Interestingly, a shift from slower growth at temperatures below 10°C to faster growth beyond 13°C seemed to occur. For example, a strain of *Acinetobacter* grew slower at 4°C than strains of

TABLE 7. *Linear correlations between the modified psychrotrophic bacteria count after incubation and the initial psychrotrophic bacteria count of manufacturing grade raw milks.**

Initial	After incubation (temperature in °C, time in h)									
	30-3	30-6	21-10	21-17	21-24	13-10	13-15	13-20	7-32	7-37
0.79	0.66	0.64	0.51	0.46	0.33	0.62	0.51	0.47	0.52	0.50

*Based on 283 milks.

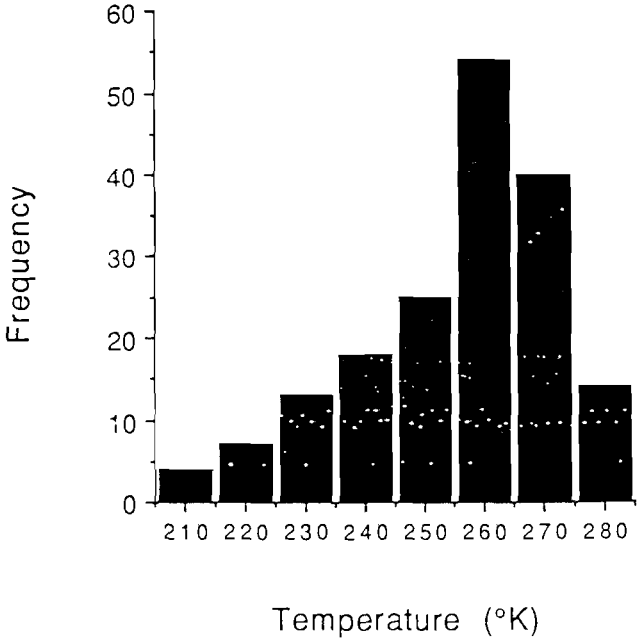

Figure 1. *Distribution of T_0 values for native flora of 283 samples of manufacturing grade raw milks.*

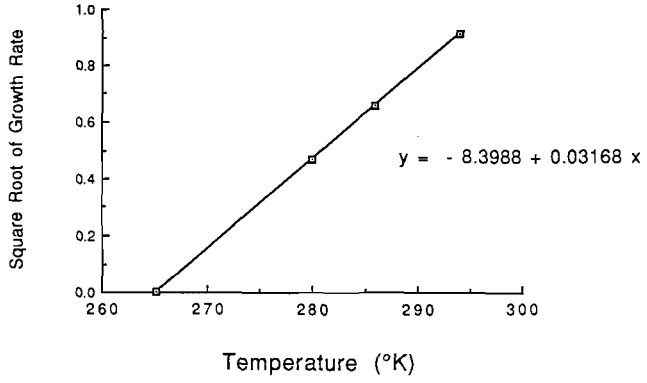

Figure 2. *Relationship between growth rate of psychrotrophs and temperature for milks with initial psychrotroph counts between 10^2 and 10^6 CFU/ml.*

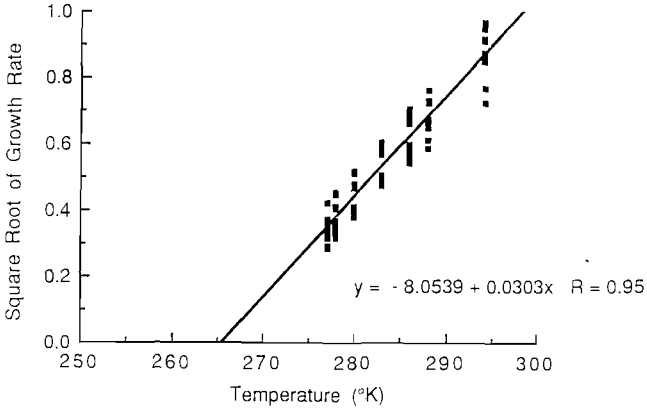

Figure 3. *Relationship between growth rate of psychrotrophs and temperature for a number of published studies (18, 24, 26, 30, 32) and our data.*

TABLE 8. *Generation times at 4, 7, and 10°C and T_0 values of psychrotrophs grown in pasteurized, homogenized whole milk.*

Organism	Generation Time (h) 4°C	7°C	10°C	T_0 value
Acinetobacter calcoaceticus	12.1	7.9	4.3	268.0
Enterobacter agglomerans	11.2	8.7	5.4	268.5
Enterobacter agglomerans	7.8	7.1	4.0	262.3
Pseudomonas fluorescens	5.8	5.9	4.0	248.2
Pseudomonas sp.	7.0	6.4	4.0	259.6
Pseudomonas sp.	5.8	4.8	3.5	256.6

Data from Terry Kirihara's M.S. Thesis, University of Minnesota, 1983. An accelerated shelf-life test to predict keeping quality of refrigerated, pasteurized milk using gas chromatography, p. 48.

either *Pseudomonas* or *Enterobacter*, but at 13°C and above the most rapid growth was exhibited by the *Acinetobacter* strain. Thus, dependent upon the nature of the component psychrotrophic flora in milk, accelerated test conditions using temperatures of 21 or 30°C will detect the rapidly growing organisms at the elevated temperatures which may not be truly representative of the microflora that flourishes at temperatures below 7°C. Likewise, incubation of plates at 21°C may detect bacteria that form colonies within 25 h, but this will not differentiate them as psychrotrophs or mesophiles. Thus, the mPBC may not distinguish psychrotrophs from mesophiles when the latter are capable of growth and colony formation at 21°C within 25 h. The correlation between mPBC and PBC will vary depending upon both the milk microflora and the preincubation conditions applied. The growth of psychrotrophic bacteria in milk can be predicted using the "square root" model proposed above. This allows estimation of the microbial population of milk at any point during storage.

When the results from five commingled grade A raw milks were analyzed, the initial PBC did not correlate well with either DEFT count or mPBC (Table 9). After storage of the milks at 4-6°C for 48 h, a significant correlation between these counting procedures was observed (Table 9). This improvement was exclusively due to the growth of psychrotrophic bacteria. DEFT counts obtained on milks preincubated at 21°C for 10 h also were strongly related to initial PBC and to PBC performed on milks stored for 48 h at 4-6°C. This relationship could be predicted using the "square root" model. Using the derived equation, generation times of the native psychrotrophic microflora of the raw milks were calculated as being 5.2 h at 6°C and 1.2 h at 21°C. Thus, the elapsed times for a nine-generation increase in count at 6 and 21°C were about 48 and 10 h, respectively. As the same level of growth was predicted, this accounts for the good correlation between counts performed on raw milks preincubated at 6°C for 48 h and at 21°C for 10 h. The DEFT system in conjunction with the use of image analyzer can be automated for handling many

samples per day. Further development of these models may prove useful for determining time/temperature combinations for more exclusive and rapid detection of PBC.

TABLE 9. *Influence of temperature on psychrotrophic bacteria count of commingled grade A raw milks and its relationship with DEFT and MPBC.*

Sample no.	Initial			After 48 h at 4-6°C			After 10 h at 21°C
	PBC	mPBC	DEFT	PBC	mPBC	DEFT	DEFT
1	$2x10^1$	$6x10^3$	$3.5x10^4$	$2x10^4$	$2.6x10^4$	$4.1x10^4$	$2.8x10^4$
2	$1.8x10^2$	$2.2x10^4$	$2x10^4$	$1.6x10^5$	$2.5x10^5$	$2.4x10^5$	$9.9x10^5$
3	$2.3x10^3$	$9.3x10^4$	$1.1x10^5$	$3.9x10^6$	$3.3x10^6$	$4.4x10^6$	$2.4x10^6$
4	$3.6x10^3$	$4.6x10^3$	$2x10^6$	$1.8x10^6$	$1.8x10^6$	$5.2x10^5$	$1.2x10^6$
5	$2.1x10^4$	$6x10^4$	$5x10^4$	$5.6x10^6$	$5.3x10^6$	$1.1x10^6$	$5.6x10^6$
Geometric average							
	$9.2x10^2$	$2.0x10^4$	$9.3x10^4$	$6.6x10^5$	$7.2x10^5$	$4.7x10^5$	$8.2x10^5$
Linear correlation between PBC and MPBC or DEFT							
	0.50	0.68		0.99	0.92	0.92	
Initial PBC and DEFT at 21°C, 10h							
							0.91

PBC: 7°C, 10 d plate count
MPBC: 21°C, 25 h plate count
DEFT: Direct epifluorescent microscopic clump count

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