QMI PROJECT under direction of Dr. Mansel Griffiths, University of Guelph (2006)

Research to Determine if the QMI® Heat-Resistant Psychrotroph Test (HRTP) Time Could Be Shortened

Objectives:

The objective of this research was to evaluate the effectiveness of the proposed test to detect levels of psychrotrophic spore-formers in milk that may cause shelf-life problems. This QMI test requires at least 24 days to perform; thus modifications to the assay conditions were investigated to reduce the time taken to achieve results. The specific objectives were:

1. To evaluate the effect of different heat treatments on activation of psychrotrophic spore-formers. Raw milk was subjected to heat treatments of 65°C for 30min, 70°C for 30min, 75°C for 20min and 80°C for 10min.
2. To determine the maximum incubation temperature that could differentiate psychrotrophic spore-formers from mesophilic spore-formers, temperatures of 7°C, 11°C and 21°C was compared.
3. To investigate sample size. The test, in its current format, requires 2L of sample, we evaluated sample sizes of 50ml, 100ml, 250ml, 500ml and 2L.
4. To determine whether oxygen permeability of the QMI bag affected germination and outgrowth of psychrotrophic spore-formers.
5. To validate the modified test.

Materials and Methods:

Milk samples

Four different samples of bulk tank raw milk from different locations were collected on a bi-weekly basis continuously over the period of four months from mid February to June. All samples were kept in an ice bath to maintain the temperature at 4°C until the samples were processed.

Freshly pasteurized homogenized full-fat milk and 2% milk samples were kindly provided by Neilson Dairy, Georgetown, ON and transported to the Canadian Research Institute for Food Safety in insulated containers with frozen insert to keep the temperature at 4°C.

Heat treatments and incubation temperature:

Different conditions of temperature and time for heat treatment were used to activate spores. These are detailed in Table 1.

Table 1: Heat treatments, incubation period and temperature used for the detection of psychrotrophic *Bacillus* spores in milk samples

Holding temp/time				Incubation temp/days
65°C/30min	70°C/20min	75°C/20min	80°C/10min	7°/3-14
65°C/30min	70°C/20min	75°C/20min	80°C/10min	11°C/3-14
65°C/30min	70°C/20min	75°C/20min	80°C/10min	21°/3-14

Sample preparation and heat treatment:

Raw milk was heat-treated using the four temperature/time combinations (Table 1) in sterile tubes of 20 ml in a water bath ensuring complete immersion of the liquid. The tubes were cooled and placed in an ice bath prior to plating. Both an automated spiral plate technique and pour plate method were used to dispense 50µl and 100µl, respectively, of heat-treated milk on Plate count agar (PCA). All plates were then incubated at 7°C for 10-14 days to detect colonies of psychrotrophic spore formers. Colony count and gram staining was done to identify gram positive *Bacillus.*

In a second experiment, 20 ml of raw milk were dispensed into sterile tubes, tubes were then heat treated at the same four temperature/time combinations prior to incubation at 7°C for 3 to 14 days. One-milliliter sub-samples were removed after 3, 5, 7, 10, and 14 days of incubation and serially diluted in normal saline before plating on Milk agar (MA) using the automated spiral plate technique. Plates were incubated at 21°C for 25 hrs, colony count and gram staining was performed.

The same procedure was repeated with the addition of two more incubation temperatures, *viz.* 11°C and 21°C, along with 7°C to optimize time to detect psychrotrophic spore-formers. Each experiment was repeated at least 3 times.

In-line samples:

Using the QMI 250ml and 2 Litre Composite Bags and Aseptic Sampler, homogenized and 2% milk samples were collected just after pasteurization from the processing plant and transported to the laboratory in insulated containers. All the samples were collected by personnel trained on QMI aseptic sampler techniques at the processing plant. Sample sizes of 50ml, 100 ml, 250ml, 500ml and 2L were evaluated for isolation of psychrotrophic spore-formers. Milk samples were incubated at 7°C and 11°C and 1ml of each sample was aseptically collected after 3, 5, 7, 10 and 14 days with the sterile syringe and needle. These sub-samples were diluted if necessary and plated on Milk Agar using the automated spiral plater. Plates were incubated at 21°C for 25hrs.

Colony count and Gram staining:

Gram staining was performed at all stages of the experiment to confirm the presence of gram positive *Bacillus.* All colony counts were transformed to $\log_{10}$ values.

Results and Discussion:

Detection of psychrotrophic spore formers in milk

When heat-treated raw milk was directly plated on PCA and incubated at 7°C for 14 days either no colonies were found or very few colonies were seen. The experiment was repeated 2-3 times (from February to March) with similar results. It has been suggested by different researchers that the incidence of psychrotrophic spore-formers in raw milk is seasonal (7). Christiansson et al. (1) showed that maximal spore counts occur during the grazing season (summer and spring), whereas minimal counts occur in winter.
Results obtained when milk samples were obtained between April to mid of the July were incubated at 7°C for 3 -14 days and plated on milk agar at 3, 5, 7, 10, and 14 days are shown in Table 2. Although colonies (2 - 3 $\log_{10}$ CFU/ml) were detected after 5 days of incubation, the count of Gram-positive bacilli increased after 10 days to approximately 4 $\log_{10}$ to 5 $\log_{10}$ CFU/ml. There was little difference in growth in milks heated at either 75°C/ 20 min and 80°C/ 10min; however counts obtained after 10 and 14 days' incubation were approximately 1 log cycle lower in milks that had been heat-treated at 70°C/20min. Hanson et al. (5) also reported activation of *Bacillus* spores at 72°C, 76°C and 82°C after 14 days of storage.

Table 2: Counts (Log CFU/ml) in heat-treated milks following incubation at 7°C

Days	65°C/30min	70°C/20min	75°C/20min	80°C/10min
3	_	_	_	_
5	_	2.61	3.60	3.09
7	_	3.41	3.48	3.60
10	-	4.06	5.17	5.13
14	_	5.60	6.39	6.60

Griffiths and Phillips (3) suggest that 28% of farm bulk tanks contain psychrotrophic spore-formers, and they also showed that the growth of *Bacillus* spp. exceeded 10^5CFU/ml after 14 days of incubation at 6°C. A study by Svensson et al. (9), which included raw milk samples from 29 different farms, showed that 25% of *Bacillus cereus* isolates from these milks were psychrotrophic.

Different incubation temperatures to optimize psychrotrophic spore formers:

Three different incubation temperatures (7°C, 11°C and 21°C) were examined to compare the rate of growth of spore-formers present in milk. It has previously been suggested that a temperature of 11°C is optimal for the differentiation of psychrotrophs and mesophiles (8). Again, there was little difference in outgrowth of spores following heat-treatment at either 75°C/20min or 80°C/10min regardless of incubation temperature (Table 3). As expected, germination and outgrowth of spores was fastest at 21°C and stationary phase was reached after about 5 days at this temperature. These results indicate that this higher temperature is unsuitable for use in the QMI test. However, similar levels of growth were achieved after 14 days at 7°C and 5 days at 11°C when the milks were subjected to a heat-treatment of 75°C/20min.

Table 3: Effect of incubation at 7°C, 11°C, and 21°C on germination and growth of spore-formers following heat-treatment of milk at 75°C/20m or 80°C/10min.

Days	75°C(7)	80°C (7)	75°C(11)	80°C(11)	75°C(21)	80°C(21)
3	-	-	-	-	3.77	3.59
5	-	-	4.31	3.0	6.40	6.55
7	-	-	5.68	5.38	6.61	7.41
10	3.03*	2.66	7.01	6.80	6.21	6.0
14	4.18	4.65	7.00	7.21	6.60	6.17

* Results are geometric means for 12 milks.

Psychrotrophic *Bacillus* spp. spores can germinate below the minimum growth temperature (T_{min}) (6) and Duffrenne et al. (2) found that strains with T_{min} ranges from ≤ 5 to 11°C can germinate at 7°C. The generation times for psychrotrophic spore formers in naturally contaminated pasteurized milk was estimated to be in the range 8 – 24 h (2). According to Griffiths and Phillips (4) ≥ 95% of spores are activated following pasteurization and maximal germination of psychrotrophic *Bacillus* spp. occurs at 15°C.

Effect of Sample size:

Different volumes (50 ml, 100 ml, 250 ml, 500 ml and 2L) of milk were collected using the QMI sampler following HTST pasteurization. The milks were incubated at 7°C or 11°C for up to 14 d. Growth occurred after 7 days and 3 d in 250ml, 500ml, and 2L samples incubated at 7°C and 11°C, respectively (Table 4); whereas growth was delayed by 2 to 3 d in the 50 and 100 ml sample volumes. Regardless of sample volume, counts were similar following 7 d incubation at 11°C and 14 d incubation at 7°C.

Table 4. Effect of sample volume on germination and outgrowth of spore-formers at 7°C and 11°C.

Time	50 ml		100ml		250 ml		500ml		2 L	
(Days)	7°C	11°C	7°C	11°C	7°C	11°C	7°C	11°C	7°C	11°C
Full-fat milk										
3	-	-	-	-	-	2.6	-	2.4	-	2.9
5	-	5.9	-	6.0	-	5.5	-	3.6	-	3.6
7	-	6.1	-	6.9	2.9	6.9	2.55	6.9	2.6	6.9
10	4.25*	6.2	4.8	6.09	5.0	7.5	3.32	6.8	3.4	6.5
14	5.0	7.2	5.1	7.2	6.0	6.8	6.0	5.3	6.0	5.7

* Results are geometric means for 12 milks.

Comparison of bags with different oxygen transmission rates

Three different polyethylene films were used to prepare bags with lower oxygen transmission rates (OTR) than the QMI bag. These films were provided by Dr. Loong Tak Lim (Food Science Dept.). Bags were made in the lab and then sealed using a heat sealer.
To determine the OTR two pieces of plastic were cut and tested on an oxygen permeability tester (Ox-Tran 2/20, Modern Control Inc., Minneapolis, MN, USA). The exposed area, temperature and relative humidity were 50 cm^2, 23°C and 83% RH, respectively.

Three different milk samples were used from different batches. Using aseptic techniques, 250ml of milk were then transferred to each of the bags, which were then heat sealed and incubated at 7°C and 11°C.
The results indicate that there was little effect of OTR on germination and outgrowth of spores at 7°C (Table 5) but growth was detected earlier in the QMI bag, which had a higher OTR, when an incubation temperature of 11°C was used (Table 5). However, the growth rate in the QMI bag was acceptable at the elevated temperature.

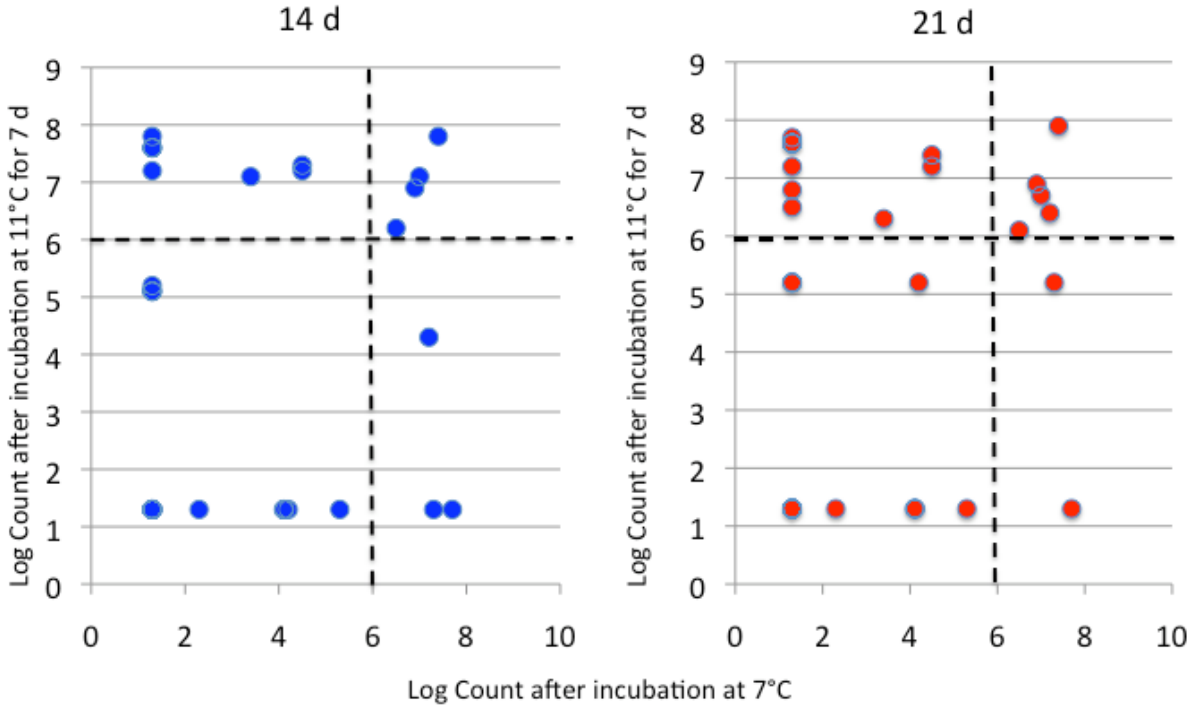
Table 5. Germination and outgrowth of spores in milk incubated at 7°C or 11°C in bags with different OTR.

	Days of incubation					**OTR**
	3	**5**	**7**	**10**	**14**	
7°C						
Bag 1	0	0	1.8	4.67	4.98	0.041
Bag 2	0	0	3.45	4.6	5.77	0.030
Bag 3	0	0	2.2	4.5	5.6	0.047
QMI bag	0	0	2.9	5.0	6.0	0.177
11°C						
Bag 1	0	0	5.7	5.8	6.7	0.041
Bag 2	0	0	5.7	6.7	6.6	0.030
Bag 3	0	0	5.7	6.7	6.7	0.047
QMI bag	2.6	5.5	6.9	7.5	6.8	0.177

Validation of assay using 11°C incubation

Milks were heat-treated and incubated at either 7°C or 11°C. The results obtained after incubation at 11°C for 7 d were compared with those obtained after incubation for 14 and 2 days at 7°C (Figure 1). The results show that using a count of 1×10^6 as being indicative of problematic levels of psychrotrophic spore-formers the modified incubation procedure correctly assigned 67% and 57% of samples after 14 d and 21 d incubation at 7°C, respectively. While these results appear to be disappointing, it should be noted that the modified incubation procedure gave only 7% (2 out of 30) false positive results. Thus, there appears to be little concern about the growth of mesophilic spore-formers at 11°C.

Figure 1. Comparison of counts obtained after incubation at 7°C for 7 days and at 11°C for either 14 or 21 days.



Validation of RS-PB-100 Poly ProBath and RS-IF-202 Incufridge

We validated poly ProBath and incufridge provided by QMI. There are three sets of experiments used to check and validate the come up time of poly probath and incufridge. We used one, two and three QMI 250 ml milk bags in different sets of experiments and also compared the results with the other water baths and incubators.
We tried the methods mentioned by QMI and we also did the same experiments with slight changes.

MATERIALS AND METHODS:

QMI composite sampling bag
RS-PB-100 Poly ProBath
RS-IF-202 Incufridge
Raytek Minitemp (infrared thermometer0
Microprocessor thermometer Type-J-K-T
Mercury thermometer
Isotemp 228 waterbath (Fisher scientific)
Isotemp 210 waterbath (Fisher scientific)

Poly ProBath was filled to the correct level with deionized water and turned on in accordance with the company's manual and guidelines. To determine the heating time, we used different thermometers for different experiments. We tried two methods; inserted the probe inside the QMI composite bag to check the come up time to 75°C of the milk itself, and the probe was also placed directly in the ProBath. We also tried an infra red thermometer as recommended by QMI to check the temperature of both the poly ProBath and Incufridge.

Table 6 shows the come-up times for the milk to reach 75°C. The heating times were found to be unacceptable and were highly dependent on the loading. Indeed when several bags were placed in the water bath the temperature of the bath decreased by 10 to 20°C.

Table 6. Come-up time to heat milk to 75°C in the ProBath

Samples	Batch1	Batch 2	Batch 3
1 bag	≥40 min	≥45min	≥55min
2 bag	1 hr 25 min	1hr 35min	1hr 25min
3 bag	1 hr 30 min	≥2 hrs	≥2hrs

We also observe the come-down time to 11°C for the Incufridge. When one heated QMI composite bag placed in the incubator it took 2 h to reach the desired temperature while it took a day when 8 QMI composite bags were placed in the incubator. Once it came down to the desired temperature it stabilized for 10 days.

Conclusions

1. A heat-treatment of 75°C for 20 min is the most suitable for processing raw milk for the QMI test.
2. An incubation time of 7 to 10 days at 11°C can be substituted for incubation at 7°C with the generation of few false-positive results.
3. Sample sizes of at least 250 ml should be processed.
4. Oxygen permeability of the QMI bag does not limit growth of the spore-formers.
5. In their present form, the PolyBath and Incufridge are not suitable for use with the QMI method.

References

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