https://doi.org/10.17221/24/2018-CJFS

Rapid Detection of Microbial Contamination in UHT Milk: Practical Application in Dairy Industry

Martina KRAČMAROVÁ[1], Hana STIBOROVÁ[1]*, Šárka HORÁČKOVÁ[2] and Kateřina DEMNEROVÁ[1]**

[1]Department of Biochemistry and Microbiology and [2]Department of Dairy, Fat and Cosmetics; Faculty of Food and Biochemical Technology, University of Chemistry and Technology Prague, Prague, Czech Republic

**Corresponding authors: kracmarm@vscht.cz (Martina Kračmarová); hana.stiborova@vscht.cz (Hana Stiborová); katerina.demnerova@vscht.cz (Kateřina Demnerová)*

Abstract

Kračmarová M., Štiborová H., Horáčková Š., Demnerová K. (2018): Rapid detection of microbial contamination in UHT milk: Practical application in dairy industry. Czech J. Food Sci., 36: 357–364.

Microbial quality of ultra-high temperature (UHT) milk is usually ascertained by a total bacterial count (TBC) cultivation. But this is time consuming, so there is a tendency to search for faster and simpler methods. We compared three instruments, focusing on shortening the detection time and their suitability for practical use in dairy plants. Two of them, RapiScreen Dairy 1000 and Promilite III, detect microbial contamination by measuring adenosine triphosphate bioluminescence; the third, GreenLight, is based on oxygen consumption analysis. In the laboratory experiments, samples of UHT milk, were spiked with low concentration of microorganisms and then the level of microbial contamination was evaluated using the above-mentioned instruments together with cultivation method as a control. The instruments were also applied in a dairy plant to test 182 real samples. All investigated methods determined microbial quality faster than the TBC, but in some cases false positive and false negative results were obtained. Therefore, precise testing including optimizing pre-incubation time for bacteria enrichment is needed prior to industrial use.

Keywords: ATP bioluminescence; oxygen-consumption analysis; total bacterial count; milk contamination

The purpose of ultra-high temperature (UHT) treatment of milk is to achieve commercial sterility through a shelf life of up to 9 months. Commercial sterility means that UHT milk does not contain any viable microorganisms and spores, which can proliferate under common storage conditions (at room temperature). Therefore, the storage of these products does not require refrigeration and enables their distribution over long distances (Griffiths 2010).

Despite the high effectiveness of heat treatment, UHT milk can show non-sterility due to two causes. The first one is by thermoduric spore-forming bacteria that live in raw milk and can survive the treatment (Boor & Murphy 2005). Typical examples of these microorganisms are *Bacillus badius*, *B. cereus*, *B. licheniformis*, *B. polymyxa*, *B. subtilis*, and *B. stearothermophilus*, lately reclassified as *Geobacillus stearothermophilus* (Pettersson *et al*. 1996; Burgess *et al*. 2017). The second way of contamination is re-introducing microorganisms into treated milk that caused the occurrence of wide range of non-spore-forming microorganisms (the most often are coliform bacteria, *Staphylococcus* spp. and *Pseudomonas* spp.). Contamination after heat treatment may happen due to a failure of the cleaning and sanitation programme (Boor & Murphy 2005). Typical spoilage sources are damaged packaging, insufficient packaging sterilization or improperly cleaned processing equipment (Fernandes 2009).

Supported by the Ministry of Education, Youth and Sport of the Czech Republic, Project No. 20-SVV/2017.

https://doi.org/10.17221/24/2018-CJFS

Microbiological analysis has an important role in UHT milk processing. The most common method of bacterial cell enumeration is total bacterial count, described in European norm EN ISO 4833-1. But a pre-incubation step has to be included before this technique can be used. During this step, UHT milk products (1 l) are pre-incubated for a few days (usually three) for bacteria enrichment. Then, the bacterial contamination in milk samples is evaluated using the pour-plate procedure. The plates are incubated for 2 days at 30°C followed by colonies enumeration. According to Council Directive 94/71/EC, milk fulfils the microbial criteria when the number of colonies is lower than 100 CFU/ml, otherwise UHT milk has to be withdrawn from sale.

During this microbial control analysis, final products have to be stored and cannot be distributed. Hence dairy processing plants are searching for a method that would provide the desired results in a shorter time. Several research teams are focused on searching for a rapid method. The most often mentioned methods are immunological or impedimetric methods (Boer & Beumer 1999; Felice *et al*. 1999), flow cytometry (Gunasekera *et al*. 2000; Holm *et al*. 2004), ATP bioluminescence (Boer & Beumer 1999; Squirrell *et al*. 2002; Carrascosa *et al*. 2012) or methods based on detection of oxygen levels (Papkovsky & Dmitriev 2013).

Measuring ATP bioluminescence is a method that is usually used in food industry not only for microbial quality control but also for evaluation of surface cleanliness (Gracias & McKillip 2004; Vilar *et al*. 2008). Even if the detection of bacterial ATP is a good microbial indicator; this method cannot identify a contaminant species or quantify the microorganisms (Carrascosa *et al*. 2012). It can only determine whether the sample is contaminated above a certain limit. Principles of ATP measurement and quantification were described previously (Griffiths 1993). The presence and concentration of aerobic and facultative anaerobic microorganisms could be also determined through respirometric analysis which is based on oxygen monitoring (O'Mahony *et al*. 2009). Since oxygen is one of the major electron acceptor of aerobic bacteria, this method is a good indicator of food microbial contaminants. Its level is monitored with fluorescence sensing where oxygen acts as a quencher. While bacterial population is growing, the oxygen level is decreasing and the fluorescence signals are less quenched and increase (O'Mahony & Papkovsky 2006). This method provides two types of output data: (i) the curve showing oxygen consumption in real-time and (ii) the time required to reach a pre-defined threshold. The threshold is defined as time when the population of microorganisms starts growing in an exponential phase and is used for bacteria enumeration (O'Mahony & Papkovsky 2006; Lehotová *et al*. 2016).

The main purpose of the present study was to compare two methods, based on ATP bioluminescence or oxygen consumption, with the classic cultivation technique (total bacterial count). The comparison was focused on: (i) the overall time required for the detection of bacterial contamination; (ii) the length of pre-incubation time of UHT milk, which is important for bacterial enrichment; (iii) the simplicity of the procedure and (iv) the accuracy of the results. Since ATP bioluminescence method is widely used in food industry, two separate instruments from different producers were tested. The microbial oxygen consumption was measured with only one instrument. They were all firstly tested under laboratory conditions on UHT milk spiked with either spore-forming or non-spore-forming bacteria. Then, their suitability for practical use in dairy processing plants was verified during one week of UHT milk production when all 3 instruments were compared on 182 real samples.

MATERIAL AND METHODS

Bacterial strains. Two different bacterial suspensions were prepared to mimic the microbial contamination in industrially processed UHT milk (contamination after heat treatment and by thermoduric strains). Hence, UHT milk samples obtained in the market were spiked with spore-forming or non-spore-forming bacteria. These strains were previously isolated from contaminated UHT milk and identified using MALDI-TOF equipment. Both suspensions were prepared at two concentrations: (i) 10 CFU/ml and (ii) 1 CFU/ml.

Suspension with spore-forming bacteria was prepared by inoculation of brain-heart infusion (BHI) medium with spore-forming *Bacillus mycoides* and incubated at 30°C. After two days, the culture was diluted to concentration of 108 CFU/ml. Finally, suspensions of 10 CFU/ml and 1 CFU/ml were prepared by several 10-fold dilutions.

The non-spore-forming bacterial suspensions were prepared differently. Four species (*Stenotrophomonas maltophilia*, *Shewanella putrefaciens*, *Citrobacter*

https://doi.org/10.17221/24/2018-CJFS

braakii and *Serratia* sp.) were incubated in BHI medium for 2 days at 30°C. The obtained suspensions were diluted to a concentration of 108 CFU/ml and mixed together in equal amounts to get a uniform representation of all the used strains. The mixture was then diluted again and suspensions containing 10 CFU/ml and 1 CFU/ml were prepared.

UHT milk inoculation, incubation and sampling. Semi-skimmed UHT milk packages (1.5% fat, 1 l) were purchased in commercial stores and inoculated, under aseptic conditions, with 1 ml of non-spore-forming or spore-forming bacterial suspension prepared in previous step. Two concentrations of bacterial suspension (1 and 10 CFU ml) were used for milk inoculation, which led to initial bacterial concentration in UHT milk 1 and 10 CFU/l, respectively. All inoculations were done in 8 replicates. Then, the inoculated packages of UHT milk were pre-incubated at 30°C. Sampling was performed immediately after inoculation and also after 24 and 48 h of pre-incubation. From each package, 5 ml was taken, vortexed and the concentration of microorganisms was analysed by: (i) total bacterial count (1 ml), (ii) ATP bioluminescence – RapiScreen Dairy 1000 or Promilite III (in both cases 2 × 50 µl) and (iii) oxygen consumption – GreenLight (2 ml).

Naturally, the equal representation of each non-spore-forming strain was less probable in UHT milk of 1 CFU/l, but we tried to simulate a failure of the aseptic process in the dairy industry followed by the contamination of UHT milk with a very low microbial concentration.

Total bacterial count. The enumeration of bacterial contamination in milk samples was determined by the pour-plate procedure using plate count agar (PCA) and two Petri dishes were used for each dilution. After the incubation of plates at 30°C for 48 h, the total bacterial count (CFU/ml) was evaluated.

ATP bioluminescence. For analysis, two different ATP bioluminometers from different producers were used: the RapiScreen Dairy 1000 (RSD; Celsis Inc., USA) and Promilite III (P III; Promicol, Netherlands). Milk samples (50 µl) were transferred to a microtiter plates of both bioluminometers. After that, the automatic measurement was triggered.

Background value of both bioluminometers had to be measured to assure correct explanation of results. The value was found by analysing 24 samples of uncontaminated UHT semi-skimmed milk (1.5%). The interpretation of results from these tested samples was carried \\\\\\\\\\\\\\out, according to the manufacturers' recommendations, as follows: a) signals that were less than double the background value were considered to be uncontaminated; b) samples with signals higher than double but lower than triple the background value needed to be repeated and c) samples with signals higher than triple the background value were considered to be contaminated.

Measurement of oxygen consumption. For the respirometry analysis, the GreenLight from Luxcel Biosciences (GL; Cork, Republic of Ireland) was used. Further information about this equipment is in Lehotová *et al*. (2016). The milk samples (2 ml) were transferred into a specific tube with a fluorescent probe and inserted into GreenLight where the oxygen level was continuously monitored at 30°C. Because the time of analysis is dependent on the initial microbial concentration (the higher microbial concentration, the shorter time to note the depletion of oxygen level), a calibration had to be performed.

To exclude the influence of different milk products and their composition, the calibration curve has to be measured using the same type of tested milk product. The calibration curve was constructed with UHT milk inoculated with the mixture of non-spore-forming or spore-forming bacteria (*Bacillus mycoides*) of following concentration: 1, 10, 102, 103, 104, and 105 CFU/ml (always in 4 replicates for each dilution). Firstly, UHT milk samples were tempered for 5 min at 30°C, secondly the oxygen level was evaluated each 5 min for 12 h with the GL instrument and also the pour-plate method was used as a control. The calibration curve was prepared by correlation of obtained results and the time required to record oxygen changes for the microbial contamination of 100 CFU/ml (legislative limit according to Council Directive 94/71/EC) was determined.If milk samples were measured longer than this time, their concentration was below 100 CFU/ml. If their measurement took a shorter time, the microbial concentration was above this limit.

Testing of all instruments in a dairy processing plant. All of the above-mentioned instruments were introduced into a microbiological laboratory in a dairy processing plant for one week. UHT milk samples (1 l) were pre-incubated 3 days at 35°C and then analysed by total bacterial count and by all the three instruments. During trial operations, 182 packages of UHT milk were analysed. In this part of the experiment, the simplicity of the instruments and their suitability for use in a dairy processing plant was evaluated.

https://doi.org/10.17221/24/2018-CJFS

RESULTS AND DISCUSSION

Assessment of instruments backgrounds. The background of the ATP bioluminescence signal, established by measuring 24 samples of uncontaminated UHT milk, were set as 8 RLU (RSD) and 11 RLU (P III). Based on these values, the correct interpretations of the obtained results were: (i) for the RSD: samples with a signal higher than 24 RLU should be considered to be contaminated, samples with 16–24 RLU should be repeated and samples with signals lower than 16 RLU are uncontaminated; (ii) for the P III: samples with signals higher than 33 RLU should be considered to be contaminated, samples with 22–33 RLU should be repeated and samples with RLU below 22 are considered to be uncontaminated.

From the calibration curve (Figure 1) of the GL, the oxygen changes of milk contaminated with at least

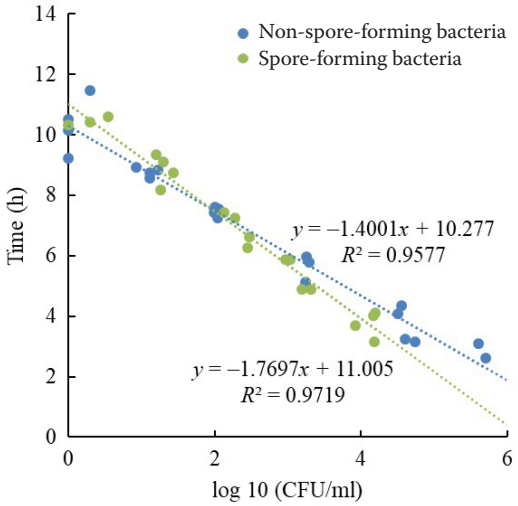

Figure 1. Correlation of total bacterial count results and by the (GL) in UHT milk samples

Non-spore-forming bacteria: mixture of *Stenotrophomonas maltophilia*, *Shewanella putrefaciens*, *Citrobacter braakii*, and *Serratia* sp.; Spore-forming bacteria: *Bacillus mycoides*

Table 1. Outputs of ATP bioluminiscence (SD, P III) RSD measurement of UHT milk contaminated with initial bacterial contamination 10 CFU/l

	Sample	0 hour			24 hours			48 hours		
		RSD signal (RLU)	P III signal (RLU)	TBC (CFU/ml)	RSD signal (RLU)	P III signal (RLU)	TBC (CFU/ml)	RSD signal (RLU)	P III signal (RLU)	TBC (CFU/ml)
Non-spore-forming	1	11	6	NC	30.465	30	3.5×10^7	9.619	32	1.7×10^9
	2	9	14	NC	25.590	53	4.2×10^7	8.836	21	1.5×10^9
	3	13	12	NC	25.009	53	5×10^7	9.714	20	6.4×10^9
	4	11	26	NC	37.397	52	8×10^7	14.126	31	2.7×10^9
	5	9	11	NC	34.939	75	1.2×10^8	16.433	30	1.8×10^9
	6	11	8	NC	37.639	71	1×10^8	12.962	30	1.3×10^9
	7	10	19	NC	47.223	67	5.2×10^8	12.344	50	2.5×10^9
	8	10	18	NC	49.463	73	2.2×10^8	21.308	27	1.8×10^9
Spore-forming	1	12	14	NC	12	10	NC	10	7	NC
	2	8	18	NC	50.379	71.155	5×10^5	60.246	71.677	1.4×10^7
	3	9	15	NC	63.950	78.266	7.6×10^5	58.715	70.351	5.6×10^6
	4	10	13	NC	51.992	69.278	4×10^5	53.305	60.522	1.3×10^6
	5	11	11	NC	17.218	10.772	6.5×10^5	40.620	43.303	9.6×10^6
	6	11	12	NC	45.131	68.608	2.5×10^5	44.087	51.526	1.4×10^7
	7	9	15	NC	49.375	78.420	2.5×10^5	52.072	6.547	2.3×10^7
	8	4	22	NC	45.982	61.703	4.6×10^5	50.787	59.375	8.5×10^6

NC – no colonies were detected on agar plates after 48 h of incubation; TBC – total bacterial counts; > no detectable changes in oxygen concentration in UHT milk samples during 24 h of measurement; 0 – changes in oxygen concentration were detected immediately; grey haighlights responses of the P III instrument which did not correspond with TBC

UHT milk samples were inoculated with spore and non-spore-forming bacteria, always in 8 replicates for each instrument; signals of bioluminescence, oxygen consumption and TBC were detected after 0, 24, and 48 h of pre-incubation time; background bioluminiscence signals were 8 RLU and 11 RLU for the RSD and P III, respectively; changes in oxygen consumption (GL) detected after less than 8 h of measurement meant that the microbial concentration was higher than 100 CFU/ml

https://doi.org/10.17221/24/2018-CJFS

Table 2. Outputs of oxygen consumption (GL) measurement of UHT milk contaminated with initial bacterial contamination 10 CFU/l

	Sample	0 hour		24 hours		48 hours	
		threshold (h)	TBC (CFU/ml)	threshold (h)	TBC (CFU/ml)	threshold (h)	TBC (CFU/ml)
Non-spore-forming	1	>	NC	0	3.5×10^7	0	1.7×10^9
	2	>	NC	0	4.2×10^7	0	1.5×10^9
	3	>	NC	0	5.0×10^7	0	6.4×10^9
	4	>	NC	0	8.0×10^7	0	2.7×10^9
	5	>	NC	0	1.2×10^8	0	1.8×10^9
	6	>	NC	0	1.0×10^8	0	1.3×10^9
	7	>	NC	0	5.2×10^8	0	2.5×10^9
	8	15.33	NC	0	2.2×10^8	0	1.8×10^9
Spore-forming	1	>	NC	>	NC	>	NC
	2	>	NC	0	5.0×10^5	0	1.4×10^7
	3	>	NC	0	7.6×10^5	0	5.6×10^6
	4	>	NC	0	4.0×10^5	0	1.3×10^6
	5	>	NC	0	6.5×10^5	0	9.6×10^6
	6	>	NC	0	2.5×10^5	0	1.4×10^7
	7	>	NC	0	2.5×10^5	0	2.3×10^7
	8	>	NC	0	4.6×10^5	0	8.5×10^6

For abbreviations see Table 1

100 CFU/ml bacteria (spore-forming or non-spore-forming) were detected after at least 8 hours. Thus, we defined this time as an upper limit for the detection of microbial concentration lower than 100 CFU/ml.

Initial microbial concentration 10 CFU/l UHT milk. After 24 h of pre-incubation of inoculated UHT milk at 30°C, the non-spore-forming bacterial concentration reached to 108 CFU/ml (verified by TBC). However, this high microbial contamination was only detected by one of the two bioluminometers (RSD) and by the GL. The signals of the P III were very low in this set of samples (Tables 1 and 2).

After 48 h of pre-incubation time, the signals of the P III decreased even more compared to those at 24 h although the microbial concentration of non-spore-forming bacteria in all these samples was 109 CFU/ml (Table 1 and 2). The values of samples Nos 2 and 3 were less than double the background value, so they could be considered as uncontaminated. The values of samples Nos 1, 4, 5, 6, and 8 were below the triple of background value, so they should be repeated according to manufacturer's recommendation.

In contrast, the set of samples contaminated with spore-forming bacteria were identified correctly by both bioluminometers. The GL, as the RSD, properly identified the microbial contamination of spore-forming and non-spore-forming bacteria immediately after 24 h of pre-incubation time (Table 1 and 2).

Initial microbial concentration of 1 CFU/l UHT milk. After pre-incubation time (0, 24 and 48 h), milk samples were measured by the GL and the RSD. The P III was not included in this part of the experiment due to its previous false negative results (Tables 1 and 2).

In this part of experiment, it is apparent that such low initial concentration of microorganisms behaved differently in case of spore-forming or non-spore-forming. Non-spore-forming bacteria were detected by the RSD and by the GL after only 24 h of pre-incubation time (Table 2). But the spore-forming bacteria were hardly detected by either instrument. After 24 h of pre-incubation, the GL detected contamination only in two samples. Nevertheless, spore-forming bacteria were not detected even by the classical cultivation method (TBC) after this pre-incubation time, which indicates that growth of spore-formers is slower compared to non-spore-formers. This phenomenon needs to be taken into account during the setting up of the pre-incubation time.

After 48 h of pre-incubation, the GL detected spore-forming bacteria in 4 contaminated samples. The

https://doi.org/10.17221/24/2018-CJFS

measurement of three samples (Table 3) was longer than 8 h, implying that the microbial concentration was below 100 CFU/ml, which was not in agreement with the TBC method, where the microbial concentration was just over the limit of 100 CFU/ml.

Similar results were obtained with the RSD. Although non-spore-forming bacteria were detected after 24 h of pre-incubation, the signals of samples with spore-forming bacteria did not correspond with their total bacterial counts (Table 3). These discrepancies were obtained after both 24 h and even 48 h of pre-incubation.

In samples No. 6, 7 and 8 the spore-forming bacteria (Table 3) were detected by neither the RSD nor the GL. Therefore, we assumed that a longer pre-incubation time would facilitate the detection of contamination when the initial concentration of spore-forming bacteria in milk is as low as 1 CFU/ ml.

Trial tests of all instruments in a dairy processing plant. Both bioluminometers and the GL were introduced into trial operation in a dairy processing plant for a week. The pre-incubation time was set up to 72 h according to dairy laboratory practise and the results are shown in the Table 3. Milk samples were then analysed with each instrument and the results were compared with total bacterial counts.

During this trial operation, 182 packages of UHT milk were tested and 4.9% of samples were determined as contaminated using the classical cultivation method. In these cases, the concentration of microorganisms never reached 105 CFU/ml (results not shown). All results nicely correlated with the ones obtained with both bioluminometers. However, the results obtained from measurement with the GL showed 2.2% false positives (4 samples). In these cases, the curves of oxygen consumption were examined in detail and compared with the curves of uncontaminated samples. The curves of false positive samples crossed the threshold at the beginning of the measurement but after 10 min of readings the signal decreased and the curves were identical to uncontaminated samples. We assume that this might have happened due to insufficient tempering of these samples.

Tested instruments provide a fast, easy and automated way for microbial analysis of UHT milk in dairy processing plant. From the results summarized in Tables 1 and 2, it is seen that both ATP bioluminometers (RSD and P III) and the GL significantly decreased the time of determination of microbial contaminants. The pre-incubation time of all these methods was the same as for the cultivation technique, therefore the main difference of overall detection

Table 3. Outputs of RSD and GL measurement of UHT milk contaminated with initial bacterial contamination 1 CFU/l

	Sample	0 hour			24 hours			48 hours		
		GL threshold (h)	RSD signal (RLU)	TBC (CFU/ml)	GL threshold (h)	RSD signal (RLU)	TBC (CFU/ml)	GL threshold (h)	RSD signal (RLU)	TBC (CFU/ml)
Non-spore-forming	1	>	10	NC	0	34 246.5	6×10^8	0	14 591.5	6.5×10^8
	2	>	9	NC	0	29 756.5	1.3×10^9	0	15 119	7.9×10^8
	3	5.9	222.5	7	1.2	4 540	4.5×10^2	0.1	12 484.5	8.3×10^8
	4	34.7	10	NC	>	8.5	NC	>	11	NC
	5	>	11.5	NC	>	11.5	NC	>	8.5	NC
	6	>	14.5	NC	0	41 754	8×10^8	0	19 219	5.7×10^8
	7	>	10	NC	0	89 410.5	1.5×10^9	0	30 340	1.3×10^9
	8	>	10.5	NC	0	57 904	1.3×10^9	0	30 564	8.2×10^8
Spore-forming	1	>	14	NC	0	68 107	1.5×10^7	0.2	26 201	3×10^6
	2	>	12	NC	9.8	9.5	$1.2 \times 10^{2*}$	6.2	14	3.2×10^3
	3	>	15	NC	>	14	NC	7.8	9.5	3.7×10^3
	4	>	12.5	NC	>	9.5	NC	9.4	9	3.5×10^1
	5	>	13.5	NC	>	12.5	NC	3.4	67	2.5×10^5
	6	>	11.5	NC	>	10.5	NC	8.2	12	1.5×10^2
	7	>	12.5	NC	>	11	NC	8.7	13	2.1×10^2
	8	>	11.5	NC	>	11.5	NC	10.3	12	1.2×10^2

For abbreviations see Table 1

https://doi.org/10.17221/24/2018-CJFS

time was in the analysis of milk samples done by the instruments. The measurement of ATP bioluminometers (RSD and P III) took 20 min and the GL about 8 h, which is a significant time reduction. But, as the results show, all the three instruments would need to be calibrated prior to their introduction into the dairy processing plant.

Respiration analyses are usually performed in real-time and it took a few hours to obtain the results (Papkovsky & Dmitriev 2013). Due to very low microbial concentration in UHT milk, the pre-incubation step must be included into the analysis. The length of this step was tested with the GL instrument after a low initial microbial contamination (10 or 1 CFU/ml). It was found that UHT milk had to be pre-incubated for 3 days at 30°C to obtain a reliable detection of spore-forming and non-spore-forming bacteria. So, the overall detection time was reduced to almost 3.5 days compared to 5 days using the classical cultivation method. This is a significant time saving. Under these assumptions, the GL is a good alternative to the TBC method and suitable for the food industry. But the calibration of the GL instrument needs to be performed by a trained microbiologist (Lehotová *et al*. 2016). When positive results are obtained, we recommend examining the curves of oxygen consumption and not just the 'contaminated' or 'uncontaminated' output of the instrument.

Moreover, this respirometric assay is more robust than flow cytometry or turbidimetry and the measurement is contact-less, so bacteria are not damaged and milk samples can be used for further identification of contaminating microorganism (Papkovsky & Dmitriev 2013). This type of analysis can be even applied in evaluating the surface efficiency of the hygienic sanitation procedure within an HACCP system (Vilar *et al*. 2008).

The pre-incubation step had to be included also in ATP bioluminescence methods. The Cunha *et al*. (2014) found a strong correlation between TBC and an ATP bioluminescence method after 48 h of pre-incubation of UHT milk samples. But in our study, we found that 48 h of UHT milk pre-incubation (at 30°C) containing a low concentration of spore-forming bacteria (< 10 CFU/l) is insufficient (Table 3). Therefore, we recommend 3 days of pre-incubation time.

Usually, the reliable function of ATP bioluminometers is tested by measuring samples with very low microbial concentration to ensure that even a very low microbial contamination would be detected. Nevertheless, RSD and P III measured much lower signals in samples with high concentration (~ 10^9 CFU/ml) of non-spore-forming bacteria (Table 1 and 2) compared with contamination of spore-formers. This could be explained by confirmed pH decrease according to previous findings (Wang *et al*. 2013), in which the highest intensity of emitted light (RLU) was found to be at a pH 7.5–7.8 and the lowest signals were obtained at a pH above 10 or below 4. However, only the RSD reliably detected microbial contamination in all samples (Table 1 and 2), no matter how high the concentration was. That indicates that the RSD probably had much stable reaction reagents compared to reagents used by P III. The P III barely detected these high spore-formers' contamination, which could cause significant problems in the food industry.

Based on the results from laboratory and trial tests, we recommended a pre-incubation time of at least 3 days to detect spore-forming and non-spore-forming bacteria. Compared to the GL, measurement with the ATP bioluminometers (RSD and P III) is shorter, it takes about 20 minutes. The disadvantage of microbial ATP detection is that the milk samples has to be mixed with chemical substances, which lysed the bacteria, so the same samples cannot be used for further identification of any contamination found.

CONCLUSIONS

We confirmed the assumption that all three tested instruments detected the bacterial contamination faster than the cultivation technique even though the pre-incubation time required for bacteria enrichment was the same. Generally, it was found that bacteria type influenced the analysis time the most. The longest pre-incubation time was required after inoculation of spore-forming bacteria because non-spore-forming bacteria proliferated faster. On the other hand, the high concentration of non-spore-forming bacteria affected the results of the P III. Even though the P III and the RSD are based on the same principle, big differences in accuracy of results were found between them. The GL detected all contaminated samples properly, nevertheless in some cases the instrument also gave false-positive results with uncontaminated samples. Therefore, we recommend careful checking of the instrument outputs including curves of oxygen consumption. The big advantage of the GL is that the samples could be further analysed, e.g. to strains identification. In conclusion, all instruments are suitable as

https://doi.org/10.17221/24/2018-CJFS

faster alternatives to the classical total bacterial count, but the precise tests have to be done prior to their usage in a milk processing plant to ensure milk quality standards.

Acknowledgement. The authors wish to thank Benjamin John Watson-Jones for English language correction.

References

Boer E., Beumer R.R. (1999): Methodology for detection and typing of foodborne microorganisms. Internation Journal of Food Microbiology, 50: 119–130.

Boor K.J., Murphy S.C. (2005): Microbiology of market milks. In: Robinson R.K. (ed.): Dairy Microbiology Handbook: The Microbiology of Milk and Milk Products. New Jersey, John Wiley & Sons: 91–122.

Burgess S.A., Flint S.H., Lindsay D., Cox M.P., Biggs P.J. (2017): Insights into the *Geobacillus stearothermophilus* species based on phylogenomic principles. BMC Microbiology 17: 140. doi 10.1186/s12866-017-1047-x.

Carrascosa C., Saavedra P., Millán R., Jaber J.R., Pérez E., Grau R., Raposo A., Mauricio C., Sanjuán E. (2012): Monitoring of cleanliness and disinfection in dairies: Comparison of traditional microbiological and ATP bioluminescence methods. Food Control, 28: 368–373.

Cunha A.F., Lage A.D., Pereira e Araújo M.M., Abreu C.F., Tassinari A.R., Ferraz M.A., Davenport K., Cerqueira M.M.O.P. (2014): ATP-Bioluminescence as a method to evaluated microbiological quality of UHT milk. Arquivo Brasileiro de Medicina Veterinária e Zootecnia, 66: 1909–1916.

Felice C.J., Madrid R.E., Olivera J.M., Rotger V.I., Valentinuzzi M.E. (1999): Impedance microbiology: quantification of bacterial content in milk by means of capacitance growth curves. Journal of Microbiological Methods, 35: 37–42.

Fernandes R. (2009): Microbiology Handbook: Dairy Products. 3rd Ed. Cambridge, Royal Society of Chemistry. 1–15.

Gracias K.S., McKillip J.L. (2004): A review of conventional detection and enumeration methods for pathogenic bacteria in food. Canadian Journal of Microbiology, 50: 883–890.

Griffiths M.W. (1993): Applications of bioluminescence in the dairy industry. Journal of Dairy science, 76: 3118–3125.

Griffiths M.W. (ed.) (2010): Improving the Safety and Quality of Milk. Volume 2 : Improving Quality in Milk Products. Cambridge, Woodhead Publishing Ltd: 97–123.

Gunasekera T.S., Attfield P.V., Veal D.A. (2000): A flow cytometry method for rapid detection and enumeration of total bacteria in milk. Applied and Environmental Microbiology, 66: 1228–1232.

Holm C., Mathiasen T., Jespersen L. (2004): A flow cytometric technique for quantification and differentiation of bacteria in bulk tank milk. Journal of Applied Microbiology, 97: 935–941.

Lehotová V., Petruláková M., Valík Ľ. (2016): Application of a new method to control microbial quality of foods based on the detection of oxygen consumption. Acta Chimica Slovaca, 9: 19–22.

O'Mahony F.C., Papkovsky D.B. (2006): Rapid high-throughput assessment of aerobic bacteria in complex samples by fluorescence-based oxygen respirometry. Applied and Environmental Microbiology, 72: 1279–1287.

O'Mahony F., Green R.A., Baylis C., Fernandes R., Papkovsky D.B. (2009): Analysis of total aerobic viable counts in samples of raw meat using fluorescence-based probe and oxygen consumption assay. Food Control, 20: 129–135.

Papkovsky D.B., Dmitriev R.I. (2013): Biological detection by optical oxygen sensing. Chemical Society Reviews, 42: 8700–8732.

Pettersson B., Lembke F., Hammer P., Stackebrandt E., Priest F.G. (1996): *Bacillus sporothermodurans*, a new species producing highly heat-resistant endospores. International journal of Systematic and Evolutionary Microbiology, 46: 759–764.

Squirrell D.J., Price R.L., Murphy M.J. (2002): Rapid and specific detection of bacteria using bioluminescence. Analytica Chimica Acta, 457: 109–114.

Vilar M.J., Rodríguez-Otero J.L., Diéguez F.J., Sanjuán M.L., Yus E. (2008): Application of ATP bioluminescence for evaluation of surface cleanliness of milking equipment. International Journal of Food Microbiology, 125: 357–361.

Wang C., Zhang B., Zhuang X. (2013): A biochemical system of rapidly detecting bacteria based on ATP bioluminescence technology. European Food Research and Technology, 236: 41–46.

Received: 2018–01–26
Accepted after corrections: 2018–10–16