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OCCURENCE AND DETECTION OF AEROBIC SPORULATING MICROORGANISMS IN RAW COW'S MILK

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ABSTRACT

The occurrence and system of detection of mesophilic and psychrotrophic aerobic sporulating microorganisms (MPAS) in raw cow's milk were studied. Samples of raw cow's milk from bulk tanks were collected 21 times from 14 farms during one year. MPAS assessment is based on inactivation of milk sample at the temperature of 80–82°C for 30 minutes followed by incubation in cultivation dishes at 30±1°C for 3 days for mesophilic aerobic sporulates (MAS), and at 6.5±1°C for 10 days for psychrotrophic aerobic sporulates (PAS). The total count of MPAS was within the span of 2.5–340 CFU/ml. Average value of MPAS was 59.4 CFU/ml, and variation coefficient was 93.1%. MPAS count for mesophilic and subsequently strictly psychrophilic microorganisms (MAS + SPAS) detected in the same incubation dishes enables to exclude overestimation of results as sporulates are capable of growing at both incubation temperatures. The average count (MAS + SPAS) was 56.9 CFU/ml representing 95.8% of the number of sums of individual dishes at two temperatures (MAS+PAS). Correlation coefficient of these two types of results (r = 0.99) provides evidence of close dependence which is expressed by linear regression equation y = 0.9773x. We can consider the method using at first 30±1°C and subsequently 6.5±1°C (MAS + SPAS) as more appropriate than the opposite order of cultivation temperatures because of better regression coefficient of linear dependence and higher correlation coefficient in relation to the sum of independent incubations (MAS + PAS).

Key words: milk; spore-forming aerobic microorganisms; mesophilic; psychrotrophic

INTRODUCTION

Spore-forming microorganisms have a special position among total microflora of milk with regard to their greatest ability to survive thermal treatment of milk and subsequently to propagate in final products (Vyletělová *et al.,* 2002; Mayr *et al.,* 1999; Abo-Elnaga *et al.,* 2002). Spore-forming microorganisms are either strictly anaerobic (SPAN) – genus *Clostridium*, which cause problems mainly in long lasting ripening of cheeses. Or they are facultatively anaerobic; it means that they grow under aerobic as well as anaerobic conditions – genus *Bacillus,* which is characteristic by a broad complex of physiological variants that are reflected in a variety of mesophilic, thermophilic and psychrophilic species (Guillaume-Gentil *et al.*, 2002; Páčová *et al.,* 2003).

During primary production of milk the spore-forming microorganisms can come from silage, soil and water. In the digestive tract of dairy cows, microorganisms are able to propagate up to 10 times, disperse in faeces and run on the body of the cow. The highest count of bacilli was found in faeces and silage, where the values exceeded 10^6 g^{-1}. Increased number of sporulates were observed in milk at the time of feed change when diarrhoea occurred in cows (Lukášová *et al.,* 2001). Significant correlations between the occurrences of spore-forming bacteria of the genus *Bacillus* in raw cow's milk and faeces (r = 0.28, P = 0.05), or in feed and faeces (r = 0.35, P = 0.01) were found when looking for ways to reduce their transfer into milk (Vyletělová, *et al.,* 2001). Statistical correlation

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analysis of total bacterial count and count of spores in raw milk from 5882 Belgian milk suppliers showed significant ($P < 0.01$) positive, although, weak ($r = 0.3$) correlation. The average counts of total bacterial and spore-forming microorganisms were $10^{4.5}$ and $10^{2.7}$ per ml milk, respectively. This means that providing low spore counts in supplied milk require the usual hygienic methods as well as further specific viewpoints and measures in basic milk production (Rombaut *et al.,* 2002).

Thermal inactivation by detection of MPAS, particularly at 80–82°C for 30 min followd by cultivation in aerobic conditions makes it possible to aim more closely at spores of spore-forming aerobic microorganisms, mostly of the genus *Bacillus*. Using two incubation temperatures - 30±1°C and 6.5±1°C, we determined the count of mesophilic as well as psychrophilic species. It is possible to include this criterion of the proposed count of maximum 200 CFU per ml milk. Round-the-year inquiry into basic production of standard quality milk in the region of dairy plant aimed at fresh milk products and milk with prolonged durability confirmed that such requirement is real (Hanuš *et al.,* 2002).

The paper deals with characteristics of microbiological contamination in milk within the year round inquiry aiming at supplementary trait of mesophilic and psychrotrophic spore-forming aerobic microflora and method of its cultivation as a means for decreasing risk and for prolonging the durability of milk-derived foodstuffs.

MATERIAL AND METHODS

In the course of one year, 21 samplings were done from bulk milk tanks of raw cow milk ready for transport to dairy plants for foodstuffs processing in 14 agricultural enterprises, equally during four seasons. A total of 294 samples were tested and Heeschen's agent was used for conservation (Heeschen *et al.,* 1969).

Total count of mesophilic microorganisms (MMC) was assessed under standard Slovak norms STN ISO 48 33 (1997) in order to confirm whether studied milk samples comply with the requirements of standard STN 57 0529. MPAS was assessed under the rule TEI 118 (methods in food science microbiology) for psychrotrophic and mesophilic aerobic bacterial spores in milk, which was developed in connection with the introduction of PURE-LAC technology in dairy plant PMV Zábřeh (Hanuš *et al.,* 2001). Basis of this process (table 1) is inactivation of the milk sample at 80–82°C for 30 minutes, inoculation of 1 ml on Petri dish and embedding in cultivation medium GTK enriched with 0.1% starch. It is also possible to incubate the inoculated dishes at 30±1°C for 3 days for mesophilic aerobic sporulates (MAS), other dishes at 6.5±1°C for 7 days for psychrotrophic aerobic sporulates or to use both cultivation temperatures for the same dishes one after the other, having such advantage that the colonies growing at both temperatures would not be counted twice into the final result. In an effort to solve this methodic question the cultivation of both contents of dishes after the first cultivation went on, after counting and labeling of colonies, in opposite way.

Results of all microbiological traits in the studied set of samples after conversion to colony forming units in 1 ml of original non-diluted milk (CFU/ml) were statistically evaluated by the software Microsoft Excel XP. Logarithmic transformation of data, which enables to represent larger capacity, was used to create the graphical representation. Calculations of regression analysis were performed from original data. We used analysis of variance to test the influence of season on levels of studied groups of microorganisms.

RESULTS AND DISCUSSION

Microbiological characteristics of studied milk

We evaluated total level of microbiological contamination of the studied milk samples in connection with limits determined by the standard STN 57 0529. Total count of mesophilic microorganisms (MMC) on average for the whole year was 38 170 CFU/ml (St. Dev = 76 600 CFU/ml). The limit of 100 thousands CFU/ml was exceeded in case of 9.2% of samples. The analysis of variance showed no statistically significant influence on medium values during the seasons of year (Kirchnerová and Foltys 2005).

Evaluation of MPAS

Mesophilic aerobic sporeforming microorganisms count (MAS) ranged from 2 to 330 CFU/ml, with average

Table 1: Scheme of assessment of microbial contamination of samples and verification of cultivation methods

	First cultivation		Subsequent cultivation	
Microbiol. group	Cultivation medium	Cultivation	Microbiol. group	Cultivation
MMC	GTK	30±1°C, 72 hrs.		
MAS	GTK+1% starch	30±1°C, 72 hrs.	SPAS	6.5±1°C, 10 days
PAS	GTK+1% starch	6.5±1°C, 10 days	SMAS	30±1°C, 72 hrs.

value of 54 CFU/ml with coefficient of variation Vk = 99.9%, geometric mean of 35.7 CFU/ml, and median of this set was 38.3 CFU/ml. More than sixty percent (61.2%) samples were in the category with counts up to 50 CFU/ml and only 3.1% samples were with counts over 200 CFU/ml. Considering that this group of microorganisms is not eliminated by thermal treatment of milk before processing to milk foodstuffs, even seemingly low MAS counts may have serious negative consequences for quality and storability of milk foodstuffs (Greifová *et al.,* 1999).

Counts of psychrotrophic aerobic sporulates (PAS) ranged from 0 to 28, on an average 5.4 CFU/ml in the studied set. Results of 63.6% samples were lower than 5 CFU/ml.

Sum of MAS and PAS counts gives total count of mesophilic and psychrotrophic aerobic sporulates (MPAS). It was within the span of 2.5–340 CFU/ml. Average value of MPAS of 59.4 CFU/ml and coefficient of variation of 93.1% indicate balanced level of this parameter. Counts to 50 CFU/ml was recorded for 55.4% samples, values not higher than 100 in 85%, and 3.1% samples had the count of MPAS higher than 200. On the basis of obtained results it is possible to support the proposal of initial limit for introduction of this parameter, namely for the count of mesophilic and psychrotrophic aerobic sporulates (MPAS) of maximum 200 CFU/ml. Hanuš *et al.* (2002) also confirmed objectivity of this limit. Such comprehensive view on purchased raw milk would be desirable for dairy plants that are processing milk for products with long durability or using specific cultures, or for baby food.

Mutual correlations of studied microbiological parameters

From the practical microbiological viewpoint it was important to observe that there is no dependence ($r < 0.1$) between MMC and MPAS in studied samples. Rombaut *et al.* (2002) also noticed the low correlation coefficient ($r = 0.3$). We see that it is of special importance to study the group of spore-forming organisms separately because of its technological and hygienic consequences. MPAS occurrence in raw milk is specific, requiring stricter criterion of hygiene at milk collection and it cannot be estimated on the basis of basic criteria of microbiological quality of milk.

For the mutual relationship between counts of psychrotrophic and mesophilic aerobic sporulates PAS and MAS, the calculated correlation coefficient $r = 0.18$ shows the need to determine psychrotrophic aerobic sporulates along with mesophilic ones because their count cannot be mutually estimated. It is valid for PAS that long storage and cooling of milk foodstuffs gives them time and living-space for propagation even also from seemingly negligible initial counts of CFU.

Influence of season

No influence of season (table 2) could be observed with any of the MAS, PAS as well as MPAS parameters by means of analysis of variance. Hygiene of milking is obviously on such level already that it is able to eliminate natural influence of different temperatures in individual seasons on the ability of microorganisms to propagate in the stable space. Individual higher values of these parameters appear as sporadically emerging faults in hygiene of milking.

Results of subsequent incubation at assessment of mesophilic and psychrotrophic aerobic spore-forming microorganisms

In dishes for assessment of mesophilic aerobic sporulates (MAS) cultivated for 72 hours at 30°±1C in a thermostat, the numbers were counted and the colonies labelled, and the dishes further cultivated at 6.5±1°C for 10 days in a refrigerator to assess the count of strictly psychrotrophic aerobic sporulates (SPAS) that did not create colonies at 30±1°C. On the contrary, dishes for assessment of PAS cultivated for 10 days in a refrigerator were subsequently cultivated for 3 days in the thermostat in order to detect the count of strictly mesophilic aerobic sporulates (SMAS) that did not grow at 6.5±1°C (table 1).

Results for SPAS varied from 0 – 28 CFU/ml, on an average 2.9 CFU/ml. It is 53.7% out of the count of separately cultivated PAS. The rest 46.3% out of the original psychrotrophic microorganism count are either mesotolerant ones, and created colonies during the cultivation at 30±1°C already, or they lost the ability of revitalization during the first incubation.

Counts for individual samples of strictly mesophilic aerobic sporeforming microorganisms (SMAS) ranged from 0.5 to 412 CFU/ml. Mean value was 52.3 CFU/ml. It constituted 96.8% of mean result of MAS.

Mean value of MPAS counts detected in the same dishes at incubation for mesophilic and subsequently psychrotrophic microorgsanisms (MAS + SPAS) was 56.9 CFU/ml, representing 95.8% of the counts detected by means of resultant sums of separately incubated mesophilic sporulates and particularly psychrophilic sporulates (MAS + PAS) which was 59.4 CFU/ml. Correlation coefficient of these two types of results ($r = 0.99$) provides evidence of close dependence which is expressed by linear regression equation $y = 0.9773x$.

Using two incubation temperatures for the same dishes in reversed order, first for psychrotrophic and subsequently for mesophilic aerobic sporulates (PAS + SMAS), the mean value of 57.7 CFU/ml was obtained, which makes up for 97.1% of the sum of independent MAS + PAS results. Coefficient of mutual correlation ($r = 0.79$) meant less close dependence which is expressed by equation $y = 0.9034x$ (fig. 1).

Table 2: Average counts of mesophilic and psychrotrophic spore-forming microorganisms in course of seasons (CFU/ml)

Season	Sample collection	PAS		MAS		MAS + PAS	
	Month/order	$\bar{x}$	stdev	$\bar{x}$	stdev	$\bar{x}$	stdev
autumn	11/1	5.14	4.94	61.1	81.4	66.3	81.1
	11/2	1.50	1.53	24.7	15.0	26.2	15.1
	12/1	1.29	0.85	30.6	29.8	31.9	29.8
winter	12/2	3.57	4.29	58.8	42.3	62.4	41.0
	1/2	9.36	4.71	88.4	97.1	97.8	98.3
	2/1	2.57	3.91	61.9	80.4	64.5	82.5
	2/2	3.61	2.68	62.0	49.5	65.6	49.1
	3/1	2.46	2.44	47.0	47.3	49.5	47.1
spring	3/2	2.79	1.64	52.1	45.4	54.9	45.7
	4/1	2.86	2.86	53.6	40.9	56.4	41.2
	4/2	3.79	5.07	54.7	61.2	58.5	61.7
	5/1	5.68	3.49	45.5	37.0	51.2	37.9
	5/2	13.18	7.66	64.0	39.1	77.2	43.6
	6/1	6.46	3.75	62.9	49.2	69.4	49.8
summer	6/2	11.50	4.00	83.3	77.2	94.8	76.6
	7/1	7.21	6.56	44.7	25.9	51.9	26.3
	7/2	8.21	5.79	28.5	14.4	36.7	15.7
	8/1	3.25	5.56	65.4	48.7	68.6	51.2
	8/2	4.29	6.55	43.1	31.0	47.4	35.5
autumn	9/1	2.68	2.55	56.5	74.2	59.2	75.3
	9/2	11.57	8.26	44.9	39.4	56.5	44.2

Although it is supposed that the sum of two separate assessments of MAS and PAS is overestimated by a certain unknown number of sporulates able to grow at both temperatures, this error is avoided with incubation of the same dishes at two temperatures and the method, the results of which are higher and approach more to this sum, can be considered as a method with better trapping and lower losses of the ability of aerobic sporulates revitalization. We can consider using the method first at 30±1°C and subsequently at 6.5±1°C (MAS + SPAS) as more appropriate because of better regression coefficient of linear dependence, higher correlation coefficient in relation to the sum of independent incubations (MAS + PAS) in comparison to the method employing 6.5±1°C temperature at first and 30±1°C subsequently (PAS + SMAS). Correctness of the method is a measure that reflects real value of the measured quantity.

CONCLUSION

Average value of MPAS (59.4 CFU/ml) and coefficient of variation (93.1%) provides the evidence of balanced level of this parameter. Only 3.1% samples had the count of MPAS higher than 200. On the basis of obtained results it is possible to support the proposal of initial limit for introduction of this parameter, namely for the count of mesophilic and psychrotrophic aerobic sporulates (MPAS) of maximum 200 CFU/ml.

Significant influence of season was not observed with any of the MAS, PAS as well as MPAS parameters by means of analysis of variance.

Use of two incubation temperatures one after another with identical set of dishes enables to exclude overestimation of results with sporulates capable of growing at both incubation temperatures. We can consider the method using at first 30±1°C and subsequently 6.5±1°C (MAS + SPAS) as more appropriate than the opposite order of cultivation temperatures because of better regression coefficient of linear dependence and higher correlation coefficient in relation to the sum of independent incubations (MAS + PAS).

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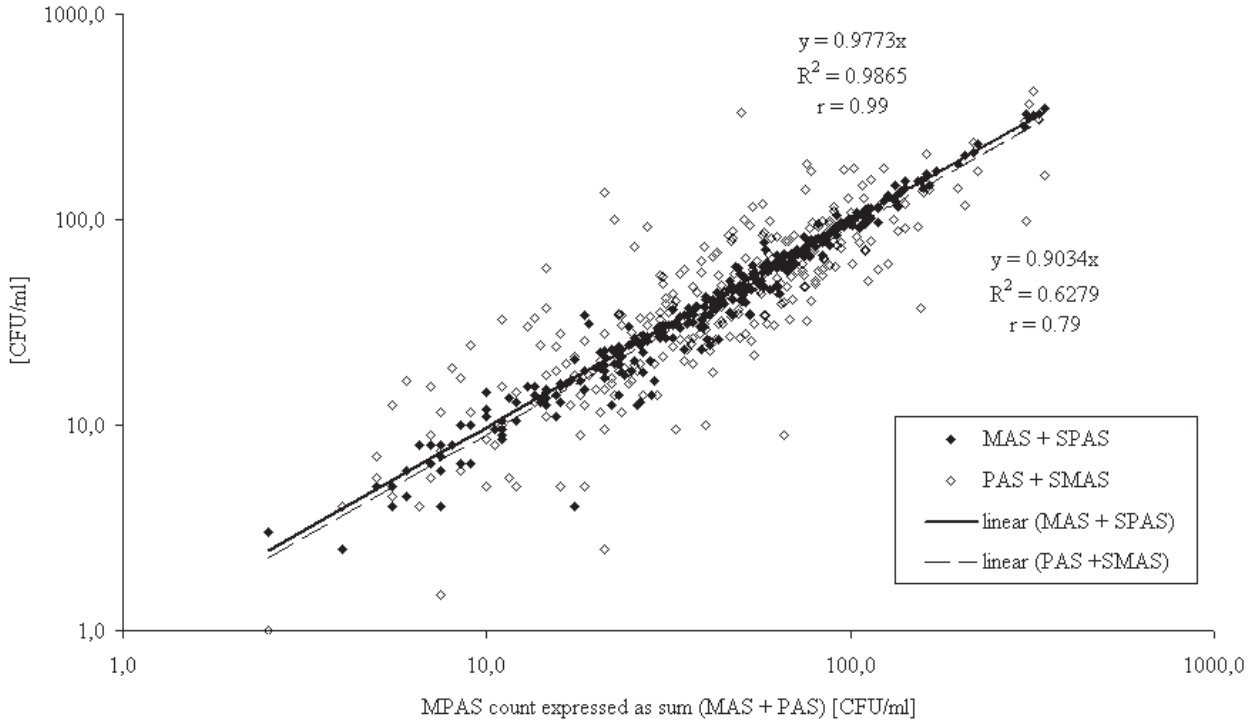

Fig. 1: Assessment of MPAS by incubation of one cultivation dish at two subsequent temperatures in relation to the sum of two dishes at two different temperatures [CFU/ml]

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