Psychrotrophic vs. total bacterial counts in bulk milk samples

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ABSTRACT: The objective of the study was to determine psychrotrophic bacteria counts (PBC) and total bacterial counts (TBC) in bulk milk samples collected during a longer period (1999 to 2000). Two sets of samples were analysed. Set 1, including samples collected in three herds, was analysed by the Central Laboratory at České Budějovice. TBC was determined using the apparatus Bactoscan, and PBC by culture according to IDF standards. Relative index p_i expressing the PBC/TBC ratio was calculated for each sample. The value of p_i for Set 1 was 0.09, ranging in herds included in this set from 0.05 to 0.20. The correlation between PBC and TBC was highly significant ($r = 0.69$; $P < 0.01$). Correlation coefficients for the individual herds ranged from 0.32 to 0.81. The correlation was highly significant ($P < 0.01$) in two herds, and significant ($P < 0.05$) in one herd. Microbiological milk quality is markedly influenced by housing and milking technologies. Another objective of this study was therefore to compare the microbiological quality of milk produced by cows housed in loose boxes and milked in parlours with that produced by cows housed in stanchion barns and milked with pipeline milking machines (Set 2). Bulk milk samples were collected and transported by standard methods and TBC and PBC were determined by culture on GTK agar supplemented with dried inhibitor-free milk according to the IDF standards. The results have confirmed favourable effects of milking in parlours on milk quality expressed in terms of counts of both technologically relevant groups of bacteria. The difference in TBC and PBC between the two milking technologies was significant ($P < 0.05$). Relative index for the milk obtained in milking parlours was $p_i = 0.18$ and that for the milk from pipeline milking machines $p_i = 0.23$. Correlation coefficients for PBC and TBC were almost identical ($r = 0.92$; $r = 0.93$; both $P < 0.01$).

Keywords: milk; total bacterial counts; psychrotrophic bacteria count; relative index; correlation coefficient

The growing role of psychrotrophic bacteria, which show metabolic activity and propagate at 4°C, in the dairy industry results from cold storage of large volumes of milk in milk rooms, and its transport to and pre-processing storage in dairies (Vyletělová *et al.*, 1999a). Psychrotrophic bacteria are becoming increasingly dangerous to the dairy industry because they produce extracellular heat – resistant lipases and proteases (Sørhaug and Stepaniak, 1991, 1997; Cromie, 1992; Champagne *et al.*, 1993; Shah, 1994). Milk altered by the activity of these enzymatic systems is depreciated and must be eliminated from processing (Sørhaug and Stepaniak, 1991; Jaspe *et al.*, 1994; Shah, 1994; Muir, 1996).

Psychrotrophic bacteria present in raw cow's milk include the Gram-negative genera *Pseudomonas, Alcaligenes, Achromobacter, Aeromonas, Serratia, Chromobacterium*, and *Flavobacterium*, and the Gram-positive genera *Bacillus*, *Clostridium*, *Corynebacterium*, *Streptococcus*, *Lactobacillus*, and *Microbacterium* (Sørhaug and Stepaniak, 1997). Although the proportion of *Pseudomonas* spp. in the total bacterial population of fresh milk does not usually exceed 10% (Meer *et al.*, 1991), this genus ranks with the most significant psychrotrophic bacteria found

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in decaying raw or pasteurised milk (Cousin, 1982; Chandler and McMeekin, 1985; Cromie, 1992). The genus includes species with the shortest generation interval at 0 to 7°C and the lowest theoretical growth temperature (–10°C), which rank its species with typical psychrophilic agents (Chandler and McMeekin, 1985; Sørhaug, 1992). *Pseudomonas fluorescens*, producing proteases and lipases, has been identified as a typical species responsible for technological troubles (Muir *et al*., 1979; Kohlmann *et al*., 1991; Fajardo-Lira and Nielsen, 1998; Vyletělová *et al*., 1999b, 2000; Hayes and Nielsen, 2000). Lipases are responsible for degradation of milk fat associated with the development of rancid and soapy flavour and occasionally somewhat bitter taste due to release of low-molecular fatty acids. Proteolytic enzymes induce degradation of casein, which is evident from greyish colour and bitter taste of milk (Vyletělová and Hanuš, 2000). Interestingly, the growth and enzymatic activities of psychrotrophic bacteria are stimulated by starter cultures of lactacidogenic bacteria. The probable cause of this phenomenon is the utilisation by lactacidogenic bacteria of peptides, amino acids and ammonia resulting from the activity of psychrotrophic bacteria and cumulating in the milk. On the other hand, free fatty acids released by the activity of *Pseudomonas* spp. can inhibit the growth of lactacidogenic bacteria (Sørhaug and Stepaniak, 1991; Shah, 1994; Jaspe *et al*., 1995).

The group of heat-resistant psychrotrophic bacteria surviving pasteurisation temperatures includes spore-forming *Bacillus* spp. producing extracellular proteases, lipases, and phospholipases (lecithinases), the heat resistance of which is comparable with that of enzymes produced by *Pseudomonas* spp. (Cousin, 1982; Meer *et al*., 1991; Matta and Punj, 1999). Markedly psychrotrophic among the milk borne bacilli is *Bacillus cereus* (Garcia-Armesto and Sutherland, 1997). Further heat-resistant psychrotrophic bacteria are classified with the genera *Arthrobacter*, *Microbacterium*, *Streptococcus*, *Corynebacterium*, and *Clostridium* (Cousin, 1982; Meer *et al*., 1991). Generation times and lag phases of psychrotrophic bacilli at 2 to 7°C are probably longer than those of *Pseudomonas* spp. (Chandler and McMeekin, 1985), but spore-forming psychrotrophic bacteria can become the predominating component of the flora found in decayed milk kept at 10°C (Meer *et al*., 1991; Stepaniak, 1991).

Safety requirements put on animal products including raw cow's milk are currently regulated by Veterinary Care Act No. 166/1999 and are specified in the appropriate implementation regulations in terms of hygienic limits for TBC (≤ 100 000 CFU/ml), expressed as geometrical mean for the last 2 months, as one of the major indicators of milk quality. PBC is used as a supplementary indicator of milk quality. The current Czech limit for PBC is ≤ 50 000 CFU/ml. Data on PBC are required by some dairies because of specific technological requirements and quality-dependent payment for raw milk supplies (Vyletělová *et al*., 1999a). The current EU standards for top quality milk require that TBC and PBC shall not exceed 30 000 CFU/ml and 5 000 CFU/ml, respectively, which corresponds to the ratio 6/1. Changes in this ratio, resulting mostly from increases in the proportions of proteolytic and lipolytic and mostly psychrotrophic bacteria in raw milk are regarded as a frequent cause of the current unexplained problems in milk processing. Data published by Kadlec *et al*. (2001) for the Czech Republic indicate that the requirement of the EU standard for TBC was met by only 50.7% and 57.56% of the samples tested in 1999 and 2000, respectively. Our two-year monitoring of three herds demonstrated that, in 1999, this requirement was met by min. 50% and max. 96.15 % of the tested samples and that the lowest proportion of satisfactory samples was found on a farm using pipeline milking machines (Cempírková and Thér, 2000). The minimum and maximum values for 2000 were 70.83% and 87.5%, respectively. The requirement of the EU standard for PBC was met by min. 37.5% and max. 66.7% of the tested samples. Again, the lowest percentage of satisfying samples was found on a farm using pipeline milking machines (Cempírková and Thér, 2000; Cempírková, 2001). Problems with meeting the EU requirement for PBC under local milk production conditions were pointed out by Vyletělová *et al*. (1999a).

Vyletělová *et al*. (1999a) found a significant correlation (r = 0.96) between TBC and PBC in a set of bulk milk samples collected concurrently in a supply area; the correlation was lower (r = 0.61) in samples collected on one farm during a longer period.

The objective of this study was to establish the PBC/TBC ratio in bulk milk samples collected

during a longer period and effects of housing and milking technologies thereon.

MATERIAL AND METHODS

The first set of samples (n = 144) included bulk milk samples collected in 1999 and 2000 in three herds (n = 48; n = 48; n = 48) monitored regularly for the PBC/TBC ratio and the relative index p_i considering the effects of housing and milking technologies (herds Ce and Hd – loose boxes, milking parlour; herd Nh – stanchion barn and pipeline milking machine). The two indicators of microbiological quality of raw cow's milk were determined in the authorised Central Laboratory of the State Veterinary Administration of the Czech Republic for milk examination – CL České Budějovice Agromléko. The samples were collected into sterile samplers containing a preservative agent (30 ml milk, 3 ml Heeschen's agent) (Heeschen *et al.*, 1969). Milk samples were transported in thermoboxes with cooling inserts, and processed immediately after delivery. TBC was determined using the apparatus Bactoscan 8000 according to the Czech Standard ČSN 57 0539 (Automated Determination of Bacteria in Raw Milk by Direct Counting of bacterial cells, January 1999) and PBC according to the Czech Standard ČSN 57 0537 (Automated Determination of Microorganism Count in Milk by Abbreviated Culture, derived from the IDF Standard 132 A: 1991 – Milk – estimation of numbers of psychrotrophic microorganisms – rapid colony count technique 25 hours at 21°C).

The second set of samples (n = 32) was used to test the effect of housing systems and milking technologies in microbial quality of milk. Bulk milk samples were collected on five farms in South and West Bohemia in 2000. Pipeline milking machines were installed on three farms (n = 21) and milking parlours on two farms (n = 18). The samples were collected into sterile preservative-free bottles, transported in thermoboxes with cooling inserts, and processed in our laboratory immediately after delivery. Total (TBC) and psychrotrophic bacterial (PBC) counts were determined. Psychrotrophic and mesophilic bacteria were grown on plates of GTK agar supplemented with dried inhibitor-free milk. The samples were diluted with sterile peptone-supplemented physiological saline. Three successive dilutions were inoculated in duplicates. The plates for the determination of TBC were incubated at 30°C for 72 h and those for the determination of PBC at 6.5°C for 10 days. Only plates with 10 to 300 colonies were considered for counting. Psychrotrophic bacteria (PPC) were enumerated after culture at 6.5°C for 10 days, while CL used culture at 21°C for 25 h. As stated by Kasalica and Otenhajmer (1995), the two methods yield almost identical counts of CFU/ml and differ only in percentages of genera of psychrotrophic bacteria. Gram-negative rods and Gram-positive cocci predominate in the results of the conventional and the shortened method, respectively.

Data processing: correlation coefficients for PBC and TBC and relative index p_i (within-sample real value ratio PBC/TBC) were calculated. In Set 2, the differences in TBC and PBC between the farms with different housing and milking technologies were assessed by the T-test. Considering the higher variability due to lack of discipline in milking, the data were transformed logarithmically for correlation and regression analyses. This corresponds to the growth of values that approaches geometric or exponential progression. Therefore, logarithmic data transformation was justified. For the same reason geometric means were calculated. The data were processed using the Microsoft Excel 97 software. Correlation coefficients, significance levels of the tested sets (TBC; PBC) and T-test of differences between TBC and PBC were calculated using the Stat Plus, Version 1.01 software.

RESULTS

Proportions of and relations between counts of technologically relevant bacteria counts in bulk milk samples

In Set 1 (n = 144), PBC ranged between 0.7×10^3 and 64.8×10^3 CFU/ml and TBC between 5×10^3 and $4\,239 \times 10^3$ CFU/ml. The relative index PBC/TBC was $p_i = 0.09$ (Table 1). The calculated regression line was $y = 0.46664x + 1.6421$. The correlation between PBC and TBC was highly significant (r = 0.69; $P < 0.01$; Figure 1).

The proportions of technologically relevant groups of bacteria (PBC and TBC) in Set 1 reflected above all hygienic standard and technological factors (Table 2). The microbial milk contamination was lower in the herds housed in loose boxes and milked in parlours (herds Ce and Hd) than in the

Table 1. General characteristics of Set 1 (n = 144)

TBC range (CFU/ml)	5 000 to 4 239 000
PBC range (CFU/ml)	700 to 64 800
Geometric mean TBC (CFU/ml)	21 558
Geometric mean PBC (CFU/ml)	4 607
Relative index p_i	0.09
Correlation coefficient r (log TBC × log PBC)	0.69

herd housed in a stanchion barn and milked with a pipeline milking machine (herd Nh). PBC in the herds Ce (n = 48) and Hd (n = 48) ranged from 0.9 × 10^3 to 39.7 × 10^3 CFU/ml and from 0.7 × 10^3 to 10.2 × 10^3 CFU/ml. The corresponding range for the herd Nh (n = 48) was from 1.4 × 10^3 to 64.8 ×10^3 CFU/ml. The hygienic limit for PBC (50 000 CFU/ml) was exceeded in only one sample (0.02%) collected in the herd Nh. The influence of housing and milking conditions on TBC was even more marked. The values ranged from 5 × 10^3 to 370 × 10^3 in the herd Ce (n = 48), from 7 × 10^3 to 83 × 10^3 in the herd Hd (n = 48), and from 5 × 10^3 to 4 223 × 10^3 in the herd Nh (n = 48). The hygienic limit for TBC (100 000 CFU/ml) was exceeded during the whole monitoring period in 20.83% of the samples collected in the herd Nh, in 6.25% of the samples collected in the herd Ce, and in none of the samples collected in the herd Hd. Relative indexes PBC/TBC for the herds Ce, Hd, and Nh were p_i = 0.19, p_i = 0.20, and p_i = 0.05, respectively.

The regression lines for the herds Ce, Hd, and Nh were defined by the equations $y = 0.5936x + 1.1153$ (Figure 2), $y = 0.6964x + 0.6932$ (Figure 3), and $y = 0.5196x + 1.4033$ (Figure 4), respectively.

Table 2. Characteristics of Set 1 for the individual herds (Ce n = 48; Hd n = 48; Nh n = 48)

Herd	Ce	Hd	Nh
TBC range (CFU/ml)	5 000 to 370 000	7 000 to 83 000	5 000 to 4 239 000
PBC range (CFU/ml)	900 to 39 700	700 to 10 200	1 400 to 64 800
TBC geometric mean (CFU/ml)	18 538	19 693	27 444
PBC geometric mean (CFU/ml)	4 454	4 283	5 126
Relative index p_i (PBC/TBC)	0.19	0.20	0.05
Correlation coefficient r (log TBC × log PBC)	0.72	0.32	0.81

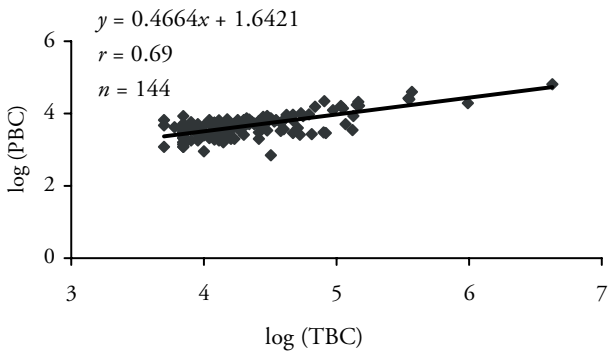

Figure 1. Correlation between TBC and PBC in samples collected in herds Ce, Hd, and Nh in 1999 to 2000

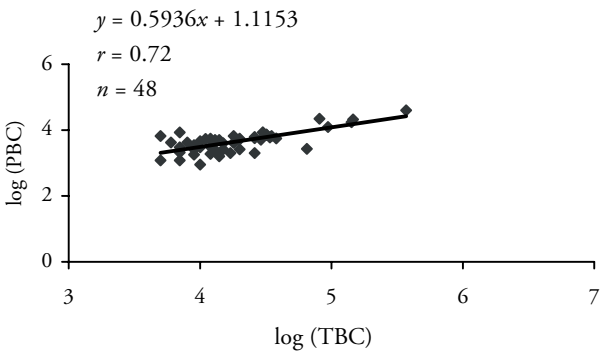

Figure 2. Correlation between TBC and PBC in samples collected in herd Ce in 1999 to 2000

The correlation coefficients within the TBC and PBC sets (n = 48) in the herds Ce, Hd, and Nh were r = 0.72, r = 0.32, and r = 0.81, respectively (Figures 2, 3, 4). The correlations were highly significant ($P < 0.01$) in the herds Ce and Nh, and significant ($P < 0.05$) in the herd Hd.

Effects of housing and milking technologies on microbial contamination of milk

Microbiological examinations of bulk milk samples collected in two herds housed in loose boxes and milked in parlours (n = 18), and three herds housed in stanchion barns and milked with pipeline milking machines (n = 21) were conducted to assess the effects of housing and milking technologies on microbial milk quality. PBC in milk obtained in milking parlours and from pipeline milking machines ranged from 0.24×10^3 to 18.00×10^3 CFU/ml and from 0.49×10^3 to 63.64×10^3 CFU/ml, respectively. The corresponding ranges for TBC were from 2.97×10^3 to 91.82×10^3 CFU/ml and from 5.32×10^3 to 148.64×10^3 CFU/ml, respectively. Limits for TBC were exceeded in 14.29% and those of PBC in 4.76% of the samples. All of them were collected in stanchion barns. The differences in TBC and PBC between the herds using different housing and milking technologies were significant ($P < 0.05$). Relative index PBC/TBC for the milk obtained in milking parlours was p_i = 0.18 and that for the milk from pipeline milking machines was p_i = 0.23. Correlation coefficient for milk samples obtained in milking parlours was r = 0.92 ($P < 0.01$) and for those from pipeline milking machines r = 0.93 ($P < 0.01$) (Table 3). Regression lines were defined by the equations $y = 1.1248x - 1.3098$ for milk samples collected in pipeline milking machines (Figure 5) and $y = 1.2142x - 1.7489$ for those from pipeline milking machines (Figure 6).

DISCUSSION

Expectably, the correlation between psychrotrophic and total bacterial counts was significant. The correlation coefficient for bulk milk samples of

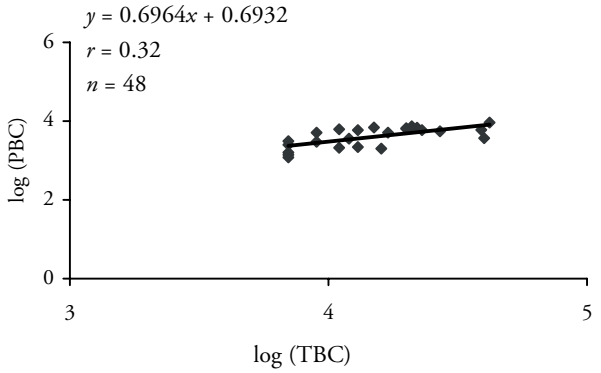

Figure 3. Correlation between TBC and PBC in samples collected in herd Hd in 1999 to 2000

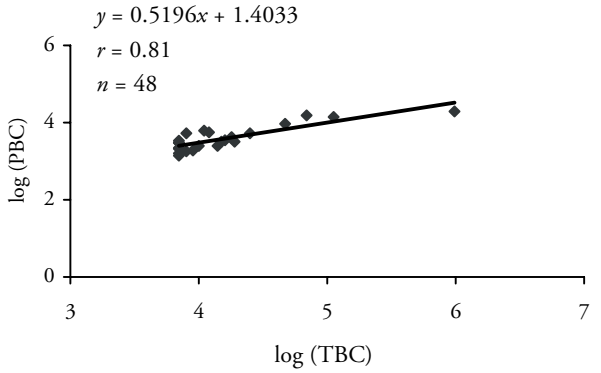

Figure 4. Correlation between TBC and PBC in samples collected in herd Nh in 1999 to 2000

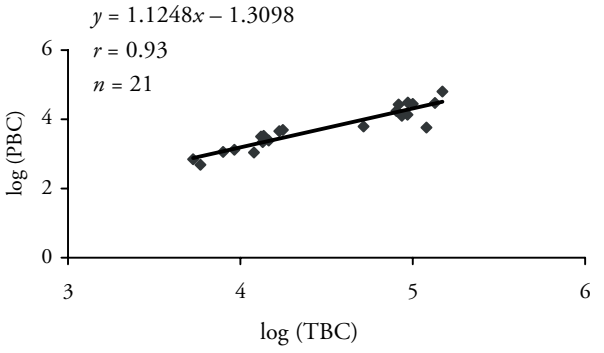

Figure 5. Correlation between TBC and PBC in herds milked in stanchions with a pipeline milking machine

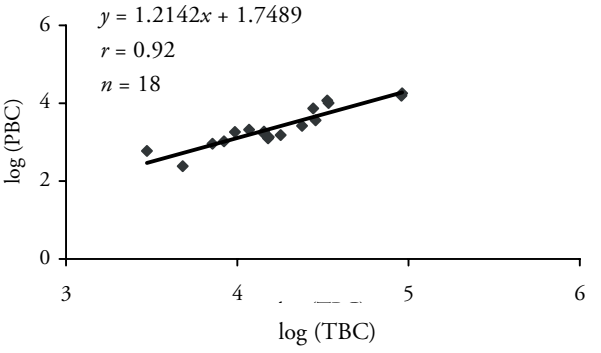

Figure 6. Correlation between TBC and PBC in herds milked in parlours

Table 3. General characteristics of Set 2

Milking in	Parlours (n= 18)	Stanchion barns (n = 21)
TBC range (CFU/ml)	2 970 to 91 818	5 318 to 148 636
PBC range (CFU/ml)	241 to 18 000	486 to 63636
TBC geometrical mean (CFU/ml)	17 050	30 785
PBC geometrical mean (CFU/ml)	2 450	5 477
Relative index p_i (PBC/TBC)	0.18	0.23
Correlation coefficient r (log TBC × log PBC)	0.92	0.93

Set 1 (current sampling for 2 years; three herds) was r = 0.69 (P < 0.01). This value is higher than that published by Vyletělová *et al.* (1999a) (r = 0.61), probably because our samples were collected regularly at shorter intervals and covered a longer period. Within-herd correlation coefficients found in our study were highly significant (r = 0.72; r = 0.81; P < 0.01) or significant r = 0.32; P < 0.05). These differences may have been due to different rates of environmental microbial contamination and herd-specific influences of the same technological factors, including milking hygiene and milk storage, on PBC and TBC (Vyletělová *et al.* (2000). This explanation is supported also by between-herd differences in relative indexes (p_i = 0.19; p_i = 0.20; p_i = 0.05).

The correlation between PBC and TBC in the samples of Set 2 was surprisingly high. The correlation coefficients for the samples of milk obtained in milking parlours and from pipeline milking machines were r = 0.92 and r = 0.93. Set 2 also included samples collected repeatedly at various times, but both PBC and TBC were determined by culture methods, while TBC in Set 1 was determined using the apparatus Bactoscan. Considering the smaller size of Set 2, the results obtained in the Central Laboratory should be regarded as more reliable.

The results of our investigations into the effects of housing and milking technologies on microbial contamination of milk corroborate our earlier findings (Cempírková and Thér, 2000; Cempírková, 2001) that a combination of housing in loose boxes and milking in parlours provides better hygienic conditions for milk production than housing in stanchion barns and milking with pipeline machines. The differences in PBC and TBC between the two technologies were significant (P < 0.05).

High correlation between PBC and TBC as demonstrated in Sets 1 and 2, might imply that TBC alone is a sufficient indicator of hygienic quality of raw cow's milk and that determination of PBC as a complementary indicator can be omitted. However, considerable effects of psychrotrophic bacteria with combined production of lytic enzymes on milk quality and possible hazard of an increase in the proportion of PBC up to 100% of total bacterial counts that can result from failures to maintain adequate transport and storage temperatures (Vyletělová *et al.*, 1999a,b) justify the necessity of monitoring of PBC, which can contribute to the explanation of possible seasonal problems in milk processing.

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