Incidence of psychrotrophic lipolytic bacteria in cow's raw milk

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ABSTRACT: The contamination of bulk samples of cow's raw milk (n = 491) by psychrotrophic lipolytic bacteria (PLiBC), total count of psychrotrophic bacteria (PBC) and mesophilic bacteria (TBC) was monitored for two years on eight dairy farms and the correlations among these groups of bacteria were analysed. An increase in TBC, PBC and PLiBC and in the values of free fatty acids (FFA) was tested experimentally in three milk samples in relation to time (analyses were done in 24-hour intervals until 96 hours) and storage temperature of milk samples (4; 6.5 and 10°C). Bacterial contamination of milk was determined by culture methods in accordance with IDF standards, the values of FFA were determined by an extraction-titration method. These mean values were determined in the set of samples (n = 491): PLiBC 659 CFU/ml, PBC 2 932 CFU/ml and TBC 18 932 CFU/ml. A high correlation was proved between values of PBC and PLiBC (r = 0.87; P < 0.001) while the correlation between TBC and PBC (r = 0.65; P < 0.001) and between PLiBC and TBC (r = 0.59; P < 0.001) was on a medium level. The proportional index p_i for PLiBC/PBC was 0.20, for PLiBC/TBC 0.03 and for PBC/TBC 0.16. In seasonal dynamics a statistically significant difference (P < 0.001; P < 0.05) between the increased values of TBC in the summer season was proved compared to the winter and spring season. The differences in the seasonal variation of PBC and PLiBC values were not significant. Experimental investigation of an increase in the values of tested parameters showed that at temperatures of milk sample storage 4 and 6.5°C TBC did not exceed the permissible hygienic value (100 000 CFU/ml) even after 96 hours while at 10°C it amounted to 90 000 CFU/ml after 48 hours and the limit for TBC was exceeded several times after 96 hours. PBC, which is not inhibited by cold storage to such a large extent, did not exceed the hygienic limit value for PBC (50 000 CFU/ml) even after 96 hours when milk samples were stored at 4°C, but at 6.5°C after 72 hours and at 10°C already after 48 hours the values 6 and 20 times higher, respectively, than the hygienic limit were recorded. A similar trend was observed in PLiBC, which exceeded the hazardous limit (43 000 CFU/ml) at 6.5°C after 96 hours and at 10°C already after 48 hours whereas at 4°C the limit value was not exceeded even after 96 hours. The content of FFA also increased in relation to the storage time and temperature of milk samples but in comparison with the increase in the tested groups of microorganisms the increase in FFA showed a higher correlation with storage time compared to storage temperature. A medium correlation was calculated between PLiBC and/or PBC and FFA content (r = 0.52; r = 0.57; P < 0.001).

Keywords: cow; raw milk; psychrotrophic lipolytic bacteria; psychrotrophic bacteria; total bacteria count; free fatty acids; lipolysis

Psychrotrophic bacteria may grow at a temperature of 7°C although their optimum temperature is higher. Rapid cooling and cold storage of raw milk favour the growth of psychrotrophic bacteria in milk (Barbano et al., 2006). They become dominant microflora during cold storage of milk and their

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extracellular enzymes, particularly proteases and lipases, contribute to the spoilage of milk products (Hantis-Zacharov and Halpern, 2007).

The contamination of cow's raw milk by mesophilic and psychrotrophic microorganisms is influenced by the health status and hygiene of dairy cows, hygiene of the environment in which dairy cows are housed and milked, methods of udder preparation and milking techniques, methods used for the cleaning and sanitation of milking machines and milk tanks, hygiene of the attendant staff (Cousin, 1982; Michel et al., 2001; Chambers, 2002; Berry et al., 2006; Cempírková, 2007). The rate of milk cooling to a required temperature and the length of milk storage are other important factors. Psychrotrophic bacteria counts after milking are related to storage temperature and time.

Vyletělová et al. (2000b) studied the growth dynamics of psychrotrophic, mesophilic and psychrotrophic lytic microorganisms in cow's raw milk in relation to time and different temperatures. Muir et al. (1978) reported that during cold storage at 6°C the total count of microorganisms increased by 1 log cycle after 48 hours and by 3 log cycles after 96 hours.

A significant correlation (r = 0.860; P = 0.013) was calculated between the population dynamics of mesophilic microorganisms and that of psychrotrophic microorganisms in cow's raw milk (Hantis-Zacharov and Halpern, 2007). The correlation coefficient 0.66 between mesophilic and psychrotrophic microorganisms in individual samples of milk was proved by Boor et al. (1998) while Vyletělová et al. (2000b) reported the correlation coefficient 0.53.

From the aspect of seasonal variability Vyletělová et al. (2000b) reported the highest values of TBC in the months of March, July and August and of PBC in July, September and October.

Regulation No. 853/2004 (2004) of the European Parliament and of the Council laid down the hygienic limit value ≤ 100 000 CFU/ml milk for the total count of mesophilic microorganisms in cow's raw milk. In additional traits of microbial quality of milk the hygienic limit value ≤ 50 000 CFU/ml milk was set down for psychrotrophic microorganisms (ČSN 57 0529, 1993). Lipolytic and proteolytic activities, supported by psychrotrophic bacteria, are not generally significant if the bacteria count does not exceed 10^6 CFU/ml (Cousin, 1982). On the contrary, Silveira et al. (1999) reported 2.7×10^4 CFU/ml as a sufficient count of psychrotrophic microorganisms for the initiation of lipolysis. Vyletělová et al. (2000b) considered the limit value 45×10^3 CFU/ml for proteolytic and/or lipolytic psychrotrophic microorganisms as a hazardous one for processing of milk to high-processed dairy products.

Unlike milk lipases, microbial lipases are thermoresistant, remaining active also after warming up. Extracellular enzymes of psychrotrophic microorganisms may resist to pasteurization (72°C for 15 s) and to UHT treatment of milk (138°C for 2 s or 149°C for 10 s Cousin, 1982; Koka and Weimer, 2001). Lipolysis results in the development of a high level of fatty acids that cause a bitter taste of dairy products, making them hardly acceptable (Ma et al., 2000; Deeth, 2006).

Enzymes present in cooled raw milk are either endogenous (i.e. coming from dairy cow) or they come from growing psychrotrophic bacteria (Ma et al., 2003). Changes in the level of FFA in milk can serve as an indirect indicator of lipolytic activity (Chen et al., 2003). The normal content of FFA in milk fat is between 0.5 and 1.2 mmol/100 g. The permissible maximum content of FFA is 13.0 mmol per kg for a churning method or 32.0 mmol/kg for an extraction-titration method (ČSN 57 0529, 1993). Vyletělová et al. (2000a) reported the FFA content of 49 mmol/kg determined by the extraction-titration method as the limit value of a hazard of lipolytic changes in the taste characteristics of milk.

Ouattara et al. (2004) stated that the lipolytic activity of psychrotrophic bacteria, normal in cow's raw milk, was species-specific and no general prognosis of FFA release could be made. Different values of PBC, given as the hazardous limit of the onset of lipolytic changes in raw milk (10^6 CFU/ml – Cousin, 1982; 2.7×10^4 CFU/ml – Silveira et al., 1999), can be explained by differences in the species representation of psychrotrophic bacteria and by the related different capacity of the production of lipolytic enzymes (Ouattara et al., 2004).

Hanuš et al. (2008) analysed the quality of cow's raw milk according to the content of free fatty acids by an automatic IR spectrometric method.

Pešek et al. (2005, 2006) studied the proportions of fatty acids in milk fat in Czech Pied and Holstein cattle kept in the Czech Republic.

The objective of the present paper was to determine the microbial contamination of bulk samples of cow's raw milk by psychrotrophic, mesophilic and especially by psychrotrophic lipolytic microorganisms and to evaluate statistically correlations among these technological groups that were stud-

ied in the course of two years. Dynamics of the growth of psychrotrophic lipolytic microorganisms, total count of psychrotrophic and mesophilic microorganisms and the level of free fatty acids were investigated experimentally in unpreserved bulk samples of raw milk stored at temperatures of 4, 6.5 and 10°C.

MATERIAL AND METHODS

From January 2006 to December 2007 the values of mesophilic bacteria i.e. total bacterial count (TBC), psychrotrophic bacteria count (PBC) and psychrotrophic lipolytic bacteria (PliBC) were monitored in bulk samples of cow's raw milk in eight cowsheds (collection places) of seven farms situated in mountain and foothills areas of Southern and Western Bohemia. A total of 491 milk samples was examined.

Bulk samples of cow's raw milk were collected into sterile samplers with Heeschen's preservative (Heeschen et al., 1969) at a 10:1 ratio (30 ml milk, 3 ml Heeschen's agent) and transported in thermoboxes with cooling pad. They were processed immediately after their delivery to a laboratory. Sterile saline with peptone was used for sample dilution. The medium tempered to 45°C was poured into 1 ml of the inoculum of the respective dilution. Samples were always inoculated by three successive dilutions, in two replications. Plate count skim milk agar (Merck) was used to determine total bacterial count (TBC) and psychrotrophic bacteria count (PBC). Incubation for TBC was run at 30°C ± 1°C for 72 hours and for PBC at 6.5°C ± 0.5°C for 10 days. Plates with the number of colonies 10 to 300 were enumerated. Tributyrin agar (Merck) was used for the culture of psychrotrophic lipolytic bacteria (PLiBC). Incubation was run at 6.5°C ± 0.5° C for 10 days. Colonies with the clear lytic zone were enumerated.

The dynamics of TBC, PBC and PLiBC growth and the content of FFA in cow's raw milk were monitored in three unpreserved bulk milk samples. An amount of 1 500 ml of bulk milk sample collected in a sterile manner was divided by 500 ml into sterile samplers and stored at temperatures of 4, 6.5 and 10°C. Microorganism count and the values of FFA of tested samples were determined on the day of sampling, and subsequently in an interval of 24 hours until 96 hours while the abovementioned identical culture conditions were used. The content of free fatty acids was determined by the extraction-titration method in accordance with ČSN 57 0533 (1993).

Arithmetic and geometric means, standard deviation, range of variation and variability were computed from the actual values of TBC, PBC and PLiBC. Proportional indexes (p_i) were determined for groups of microorganisms: they were calculated as the ratios of real values (PBC/TBC; PLiBC/PBC; PLiBC/TBC). Statistical evaluation

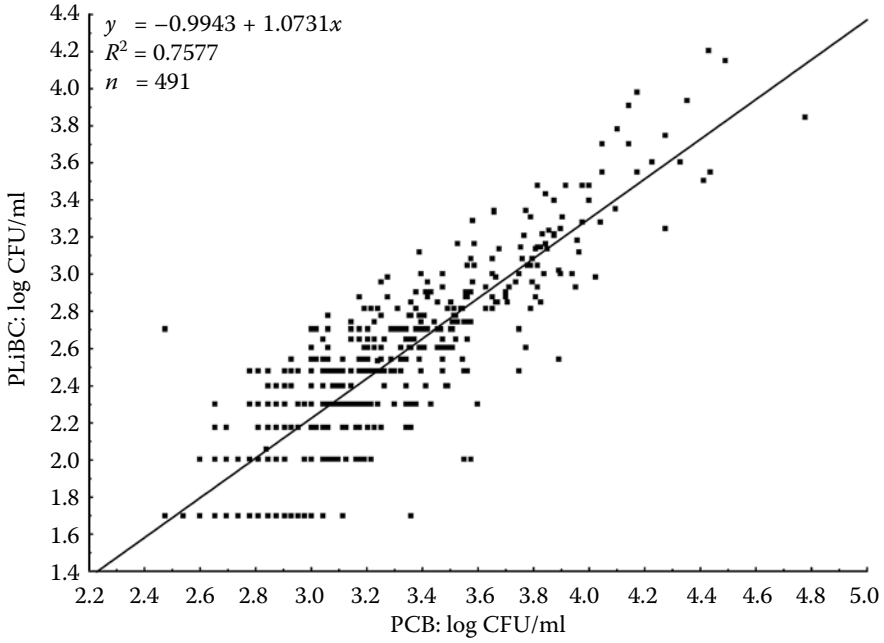

Figure 1. The linear regression relationship between PBC and PLiBC
PBC – psychotropic bacteria count
PLiBC – psychotropic lipolytic bacteria

of data was done by the Statistica CZ 7 software. Before the statistical analysis was done, the particular values of TBC, PBC, PLiBC and FFA were transformed logarithmically in order to provide for normal distribution. To evaluate relations among the studied parameters, correlation coefficients (r) and regression coefficient R^2 were computed and linear regression equations were constructed. Microbial contamination of milk in relation to the year season was evaluated by Tukey's test.

RESULTS AND DISCUSSION

The count of psychrotrophic microorganisms in the whole set (n = 491) ranged from 3 × 10^2 to 6 × 10^4 CFU/ml and the value of arithmetic mean was 2.93 ± 4.59 × 10^3 CFU/ml. The mean value of psychrotrophic lipolytic bacteria was 6.59 ± 13.8 × 10^2 CFU/ml while the values ranged from 5 × 10^1 to 1.6 × 10^4 CFU/ml. The total bacterial count showed the range of variation from 3.5 × 10^3 to 1.66 ×

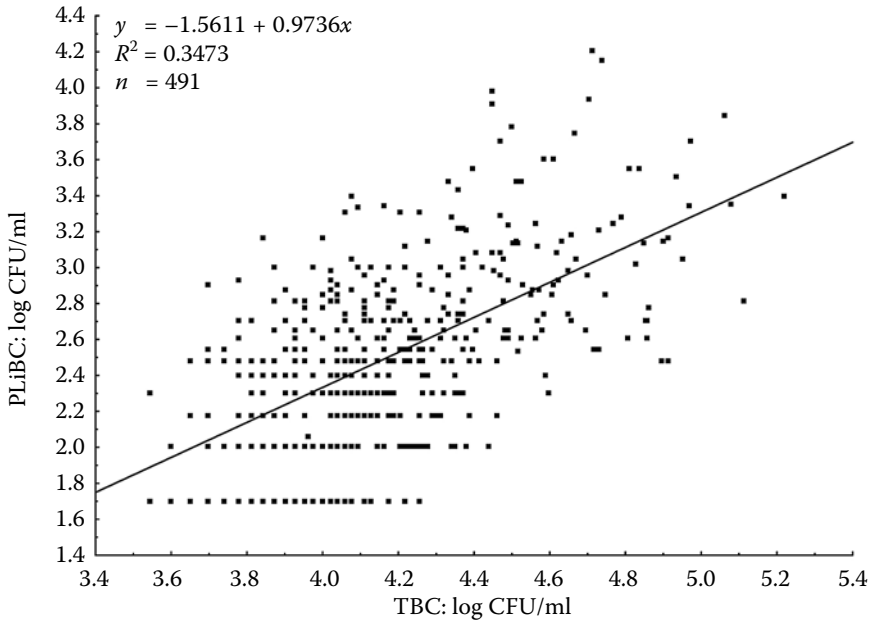

Figure 2. The linear regression relationship between TBC and PLiBC
TBC – total bacteria count; PLiBC – psychotropic lipolytic bacteria

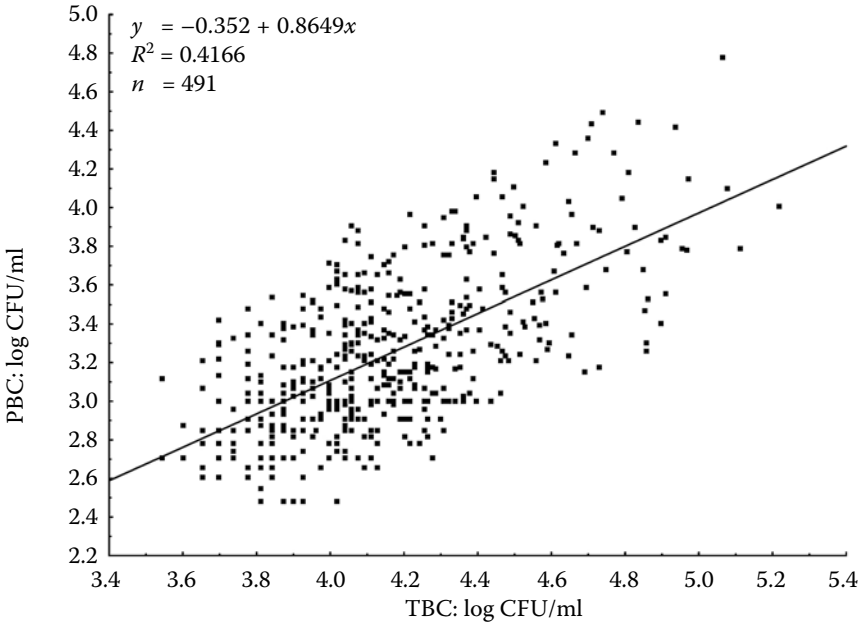

Figure 3. The linear regression relationship between TBC and PBC

10^5 CFU/ml, the mean value being 1.89 ± 1.87 × 10^4 CFU/ml. These values of microbial contamination of milk by psychrotrophic and mesophilic organisms are slightly lower compared to the preceding set (n = 365; arithmetic means of PBC 4.2 × 10^3, PLiBC 8.8 × 10^2, TBC 2.4 × 10^4; Cempírková, 2007), following the national trend of a reduction in microbial contamination of milk and representing the present farming conditions. The count of psychrotrophic microorganisms accounted for 15.5% of the total mesophilic microflora (related to the arithmetic means of the values of PBC and TBC), which is a higher percentage than that reported by Cousin (1982) when the count of psychrotrophic microorganisms in hygienic conditions is < 10% of total microflora.

Statistical evaluation of the relation between PBC and PLiBC resulted in the equation of the regression line $y = -0.9943 + 1.0731x$ (Figure 1), correlation coefficient r = 0.87 ($P < 0.001$) documenting a high correlation and proportional index p_i = 0.20. Evaluation of the relation between TBC and PLiBC gave the equation of the regression line $y = -1.5611 + 0.9736x$ (Figure 2), correlation coefficient r = 0.59 ($P < 0.001$), i.e. medium degree of correlation, and proportional index p_i = 0.03. In agreement with previous findings (Vyletělová et al., 2000b) the correlation coefficient of log PBC × log PLiBC was markedly higher (r = 0.87) than that of log TBC × log PLiBC (r = 0.59), which confirms a close relation of the technologically hazardous group of PLiBC to the microbial group of PBC, the development of which is less inhibited in conditions of cold storage of raw milk than in TBC. The value of the proportional index p_i = 0.03 for PLiBC/TBC is identical to the value reported for this relation by Vyletělová et al. (2000b) but the proportional index p_i = 0.20 for PLiBC/PBC is higher than that given by the cited authors (p_i = 0.06) for these groups of microorganisms. It can be explained by the fact that the lipolytic activity of psychrotrophic bacteria in raw milk is species specific (Ouattara et al., 2004) and the compared sets of samples originated from microbially different environments. The relation between TBC and PBC in the studied set is described by the equation of the regression line $y = -0.352 + 0.8649x$ (Figure 3), correlation coefficient r = 0.65 ($P < 0.001$) and proportional index pi = 0.16. The value of the correlation coefficient for log TBC × log PBC is almost identical with the value of the correlation for these groups of microorganisms reported by Boor et al. (1998) and Vyletělová (2000b) but it is lower than reported by Hantis-Zacharov and Halpern (2007).

As for the seasonal variation of values of microbial contamination of raw milk, increased values of TBC were recorded in summer months (June to August) while the increase in TBC values was more marked in 2006 compared to 2007 (Figure 4). The difference in TBC values in summer months and in comparison with spring and winter months was statistically significant ($P < 0.05$; $P < 0.001$). Seasonal variation did not influence other indicators. Increased values of TBC in summer months and in March were also reported by Vyletělová (2000b), but we did not observe any increase in TBC values in March. Compared to TBC the values of PBC and PLiBC did not show any marked variations in the course of 2006 and 2007 and the seasonal differences in PBC and PLiBC levels were not significant.

As expected, the dynamics of TBC, PBC and PLiBC growth and FFA content in cow's raw milk,

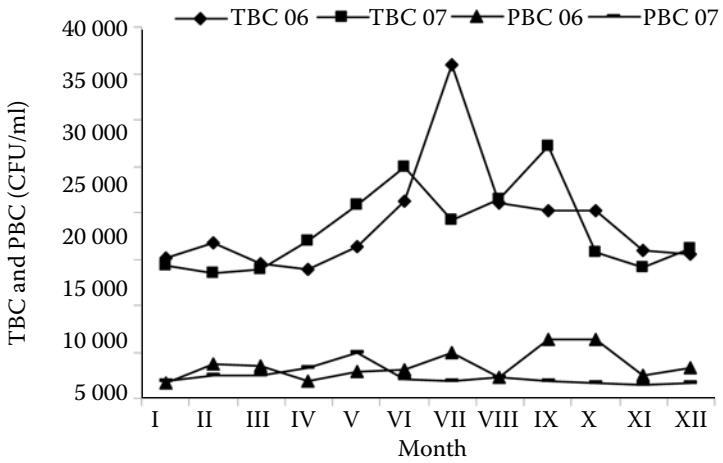

Figure 4. Dynamics of the mean values of TBC and PBC (CFU/ml) in 2006 and 2007 (n = 491)
TBC – total bacteria count;
PBC – psychotropic bacteria count

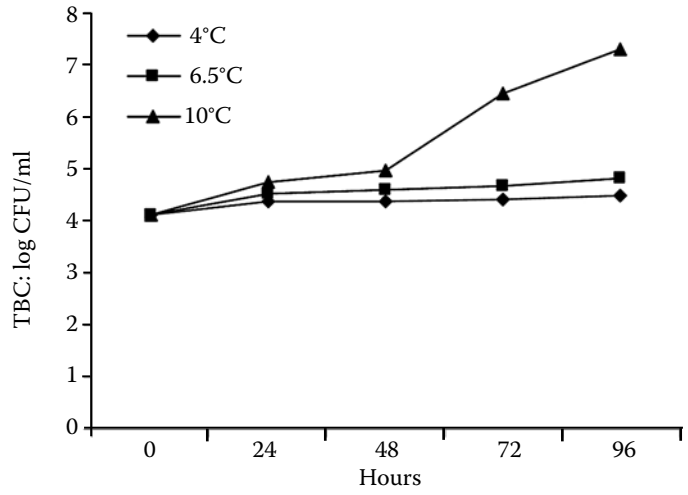

Figure 5. TBC increase related to temperature variations (the points of the graph represent mean logarithms of the values determined in three different milk samples)
TBC – total bacteria count

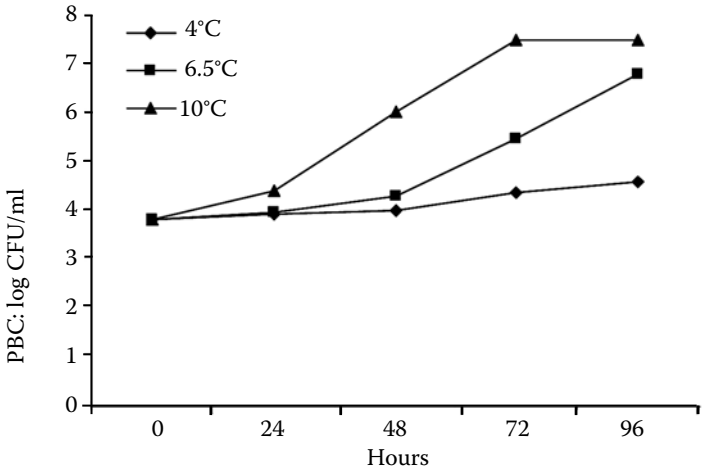

Figure 6. PBC increase related to temperature variations (the points of the graph represent mean logarithms of the values determined in three different milk samples)
PBC – psychotropic bacteria count

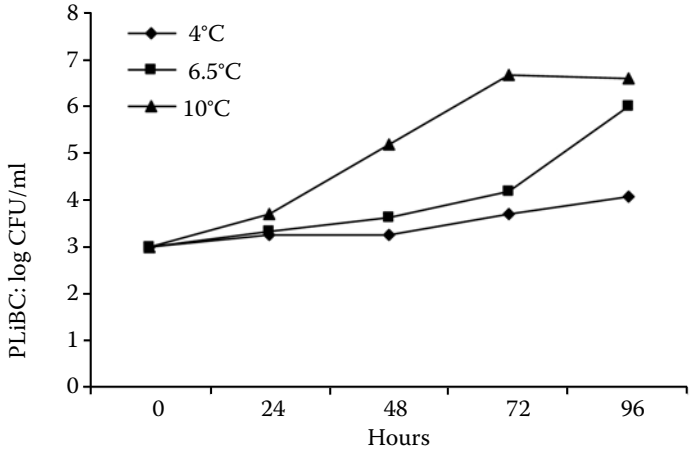

Figure 7. PLiBC increase related to temperature variations (the points of the graph represent mean logarithms of the values determined in three different milk samples)
PLiBC – psychotropic lipolytic bacteria

experimentally studied in three unpreserved bulk milk samples stored at different temperatures (4, 6.5 and 10°C), was related to time, storage temperature and to the value of the initial contamination of raw milk. The increase in TBC values was markedly inhibited by temperatures of 4°C and 6.5°C and did not exceed the admissible hygienic limit (≤ 100 000 CFU/ml) even after 96 hours. At a temperature of 10°C the values of TBC increased to 90 000 CFU/ml after 48 hours, and the hygienic limit

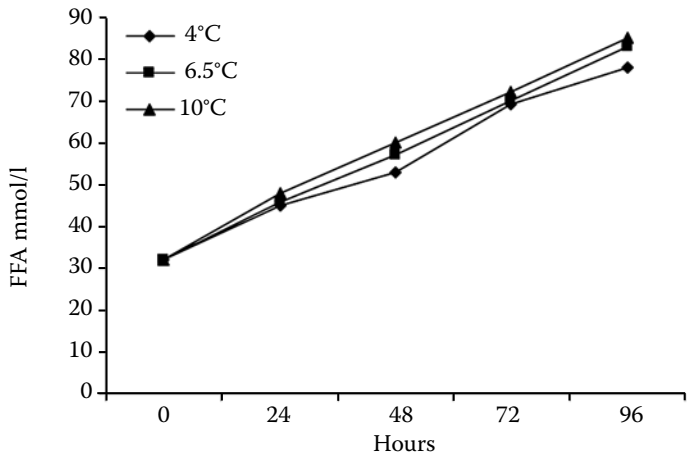

Figure 8. FFA increase related to temperature variations (the points of the graph represent mean logarithms of the values determined in three different milk samples)
FFA – free fatty acids

was exceeded several times if the samples were stored at 10°C for 72 hours. Compared to the increase in TBC in PBC and PLiBC, which are not so markedly inhibited by the cold storage of raw milk, the admissible hygienic limit for PBC (≤ 50 000 CFU/ml) was exceeded at a temperature of 6.5°C after 72 hours and at a temperature of 10°C already after 48 hours. In PLiBC, for which Vyletělová (2000b) reported 45 000 CFU/ml as the hazardous limit for higher-processed dairy products, this limit was exceeded at a temperature of 6.5°C after 96 hours and at 10°C already after 48 hours. The time onset of more marked changes in microbial contamination of milk in the course of the experiment was somewhat delayed compared to data of Vyletělová et al. (2000b), which is related with the lower initial microbial contamination of the raw milk samples we tested. Parallel investigation of an increase in the content of FFA, the level of which was 32 mmol/kg fat at the beginning of the experiment, i.e. complying with the limit of the permissible maximum content of FFA for the extraction-titration method (ČSN 57 0529, 1993), showed that the increase in FFA was also connected with storage temperature and with the time of sample storage. As the limit of a hazard of lipolytic changes in taste properties of milk Vyletělová et al. (2000a) reported the FFA content 49 mmol/kg fat if determined by the extraction-titration method. This limit was exceeded in the studied milk samples identically at all temperatures after 48 hours (FFA values in mmol/kg at 4°C = 53; 6.5°C = 57; 10°C = 60). Compared to an increase in PBC and PLiBC (Figures 6 and 7) the intensity of FFA increase (Figure 8) was in a closer relation to the storage time of milk sample than to the storage temperature at which the milk

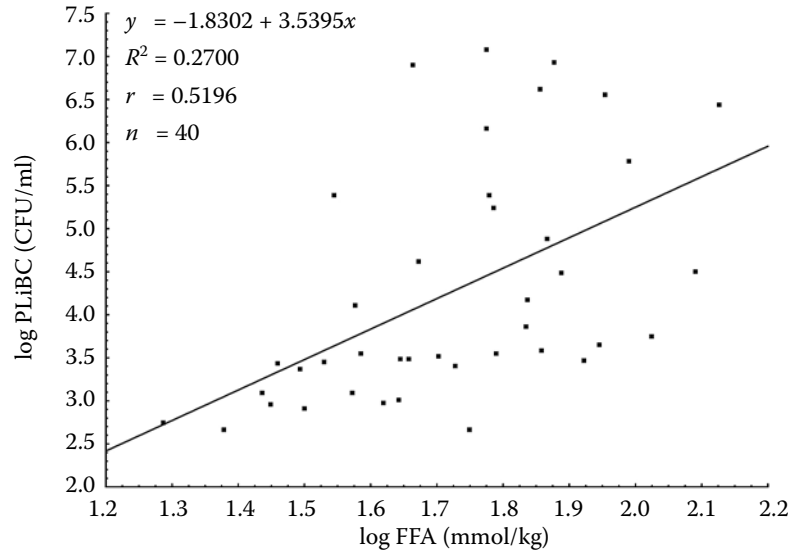

Figure 9. The linear regression relationship between PLiBC and FFA
PLiBC – psychotropic lipolytic bacteria
FFA – free fatty acids

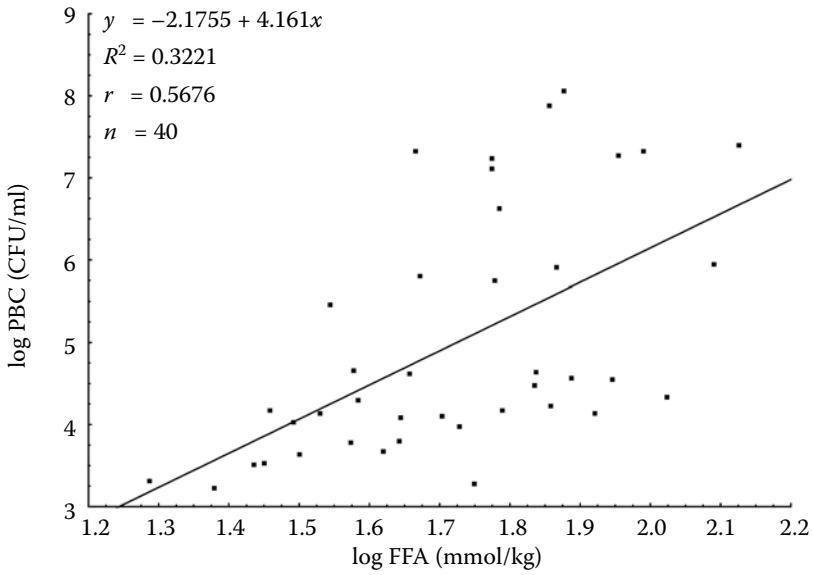

Figure 10. The linear regression relationship between PBC and FFA
PBC – psychotropic bacteria count
FFA – free fatty acids

samples were kept. Linear regression was used for statistical evaluation of the relation between PLiBC and/or PBC and FFA. The relation between PLiBC and FFA resulted in the equation of the regression line $y = -1.8302 + 3.5395x$, the value of correlation coefficient was $r = 0.52$; $P < 0.001$ (Figure 9); the equation of the regression line for the relation between PBC and FFA was $y = -2.1755 + 4.161x$ and correlation coefficient was $r = 0.57$; $P < 0.001$ (Figure 10). Contrary to the previous finding of Hanuš et al. (2008), who proved the correlation coefficient between psychrotrophic bacteria and content of free fatty acids $r = 0.27$; $P < 0.05$, i.e. a low degree of correlation, we proved a medium degree of correlation ($r = 0.52$; $r = 0.57$; $P < 0.001$) between the content of FFA and PLiBC and/or PBC. With the increasing level of PLiBC and PBC the values of FFA also increased, so it is to assume that microbial lipolytic enzymes participated in lipolysis (Ma et al., 2003). Whereas the development of psychrotrophic bacteria was more closely related with time and storage temperature, the development of FFA was related mainly with time and to a lesser extent with storage temperature. The percentage increase in FFA values at a storage temperature of 4°C ranged from 41% after 24 hours to 144% after 96 hours, at 6.5°C from 43% to 159% and at 10°C from 50% to 166%. Native milk lipases and other factors undoubtedly played their role in the degradation of milk fat (Ma et al., 2000; Deeth, 2006); therefore we consider a more detailed evaluation of the relation between FFA content and PLiBC and these other factors as significant and it will be the objective of a further study.

CONCLUSION

From the aspect of the inhibition of an increase in psychrotrophic bacteria, and mainly in psychrotrophic lipolytic bacteria in conditions of cold storage of raw milk the temperature of 4°C seems optimum as it markedly inhibits the growth of mesophilic and psychrotrophic bacteria and at the same time the increase in the values of free fatty acids is slower at this temperature compared to the temperatures of 6.5 and 10°C.

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