

EXAMINATION OF THE BIOCHEMICAL CHARACTERISTICS OF BACILLI ISOLATED FROM RAW MILK

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A total of 114 samples of raw milk were examined for the presence of bacilli. It was found that the level of bacillus contamination in raw milk depended on the place where the samples were taken. Raw milk on the farms was contaminated with bacilli at the average level of 0.92 log, while at collection points the milk contained bacilli at the level of 1.56 log and in the dairy it was also 1.56 log/ml. Bacilli were isolated after warming the milk for 10 min. at 80°C, inoculating a nutrient culture medium and incubation for 48 to 72 hours at 30°C. Bacillus ability to grow in psychrotrophic conditions was determined by storing the thermally treated milk samples for 10 days at the temperature of 7°C. Bacillus multiplication in psychrotrophic conditions varied so the milk samples could be divided into five groups. In the first group it was noticed that bacillus number decreased in psychrotrophic conditions from log 1.57 to log 0.72 which represented a highly significant environmentally influenced difference as determined by the t test. In the second group of milk samples there were bacilli that did not grow and survive in low temperature conditions, and after the milk samples were stored for 10 days at 10°C, the number of bacilli fell to less than 1/ml of milk. In the third group of samples bacilli did not show differences in number before and after storing in psychrotrophic conditions. Bacilli grew well at 7°C in milk samples placed in the fourth group and adapted to the environmental difference which was shown by the t test to be very significant. The fifth group also exhibited the appearance of psychrotrophic strains because these were detected milk stored psychrotrophic under conditions.

Examination of the ability of bacillus to grow depending on the type of medium indicated that out of 135 strains isolated, 133 grew in broth at the temperature of 45°C, 121 strains grew at 55°C, 125 bacillus strains grew in the presence of 7% NaCl and 132 strains grew in broth at pH 5.7. More than half of the examined strains (67.40%) were lecithin positive and 46.15% strains were haemolytic, which indicates the possible presence of bacilli pathogenic for people. Determination of

enzyme activity of the isolated bacillus strains, which may affect production processes in the dairy industry, showed that 40,0% degrade starch, 5.92 gelatin and 80.74% of the strains hydrolyzed casein.

Key words: bacilli, raw milk, thermally treated milk

INTRODUCTION

Reduction of the number of microorganisms in milk after thermal treatment is a function of time and temperature. Preservation of thermally treated milk depends on the number of microorganisms which survive after the thermal treatment. It is known that vegetative forms are more sensitive to temperature than spores, so it may be expected that bacillus spores or clostridia can be found even in thermally treated milk. The presence of bacilli in raw milk and final products is a risk for human health, because some strains produce enterotoxins which cause vomiting and diarrhoea. Also some bacilli cause spoiling of the products during storage, because they can grow at low temperatures and so cause economic losses.

During the last few years the ways in which fresh milk is contaminated by bacilli have been specially investigated. It been found that certain strain dominate in fresh milk.

The pathogenic activity of some bacillus strains and their ability to grow at low temperature as well as the thermostability of spores, increases the significance of bacilli detected in raw milk and final products. It has been shown that UHT products kept at 10 and 21°C for seven days contain metabolism products. Thus, products contaminated with *B. cereus* could be a risk factor for human health (Garcia et al., 1994). Storage of pasteurized milk at 5°C allows growth of *B. polymyxa* which was found in 26% samples. Storage of pasteurized milk at 7°C is more favourable for growth of *B. cereus* which was detected in 21.5%, and *B. polymyxa* in 19% samples. The presence of potentially pathogenic *B. cereus* in pasteurized milk is significant for food hygiene (Molin 1994). Conditions influencing growth and enterotoxin production of psychrotrophic *B. cereus* include water activity and temperature. The 7 strains of *B. cereus* isolated showed the presence of enterotoxin, which was confirmed by cytotoxic tests on human embryo lung cells (HEL). There was a linear connection between HEL cytotoxic titre and the number of *B. cereus* (Christiansson, 1993). Certain bacillus strains (*B. cereus*, *B. subtilis*, *B. polymyxa*) have the ability of producing the bioamines, histamine and tyramine, when thermally treated products are stored in conditions of raised temperatures (Mijačević et al., 1985).

Beside *B. cereus*, *B. licheniformis* is an ubiquitous microorganism responsible for spoiling thermally treated milk can be isolated from milk and milk products at all stages of production (Crielly et al., 1994). The proteolytic activity of bacillus species is expressed in regard to milk casein especially β -casein, releasing bitter peptides and influencing the taste of products (Babic et al., 1985; Feijoo et al., 1985; Feijoo et al., 1990; Hassan et al. 1993). It has been also determined that

B. cereus has a significantly greater proteolytic activity than other isolates of *Bacillus* spp. (Meer et al. 1993). Some bacillus proteases can be used in milk coagulation (Hassan et al., 1993; Puhan, 1996) or in the process of accelerated cheese ageing (Shehata, 1995; Marino et al., 1995; Degheidi et al., 1996). The level of bacillus contamination in foods ranges from 10^2 - 10^6 /ml (Giffel et al., 1996; Rangasamy et al., 1993; Stojanović, 1989; Mijacević et al., 1985). During investigations of raw milk psychrophilic microflora, many *Bacillus* spp. were found (Garcia-Armesto, 1994), and the level of contamination ranged from 10 - 10^4 /ml (Meer et al., 1993). The results showed that the presence of psychrotrophic bacilli in fresh milk was directly connected to the farm of origin as the most important source of this group of microorganisms, and that numbers depended on the season (Rodriguez et al., 1994). The presence of other kinds of bacillus in fresh milk also shows seasonal variations and directly depends on the way of keeping the animals. Contamination of bulked milk with *B. cereus* was the greatest in the pasturing period (Slaghius et al., 1995). Thus, cattle feed, water, faeces and samples from the udder showed that the contamination level of the environment and milk, with psychrotrophic bacteria, ranged from less than 10 up to 10^7 /g or ml for vegetative forms or less than 10 to 10^5 /g or ml for spores (Giffel et al., 1995).

Besides the original contamination, storage conditions of milk and milk products also influence their content of bacilli. When milk and milk products were kept at 7° up to the warranty period, 120 out of 458 samples, had a number of *B. cereus* greater than 10^3 /ml. The contamination with *B. cereus* in different products was similar, but germination was quicker in thermally treated cream.

Examination of raw milk after heating at 80°C for 8 min, and storage for 8 days at 7°C , showed the presence of *B. cereus* in 29 out of 115 samples (Larsen et al., 1994). *B. cereus* is usually determined as a psychrotrophic *Bacillus* genus (Sutherland et al., 1994), and it is the most frequent contaminant in food (Giffel et al., 1996). The presence of bacilli in food has been investigated as a factor of spoiling and a cause of gastroenteritis.

Thermoresistance of mesophilic bacillus spores is expressed as the time of decimal reduction (D-value). The D-value for bacilli at pasteurization temperatures ranged within several minutes and suggests the possibility of their surviving the thermal treatments (Stojanović, 1989; Ivanović et al., 1990; Oljačić, 1993, Oljačić, 1995). Spores and some bacillus strains can survive even brief thermal sterilization and be found in commercially sterilised UHT milk (Hammer et al., 1995).

The presence of bacilli in fresh milk represents a potential risk to human health and causes great commercial losses if spoiling of a product occur. There is no unique and accepted methodology of bacillus isolation from food or for estimating their pathogenicity, but the contamination level, contamination sources and bacillus thermostability has been thoroughly discussed, as well as the critical level of population necessary for producing a relevant quantity of enterotoxins.

The aim of this work was to determine the level of milk contamination by bacilli, the possibility of bacillus growth in psychrotrophic conditions as well as their biochemical characteristics.

MATERIALS AND METHODS

The material for investigation was 114 samples of fresh milk. Out of this number, 89 samples were taken immediately after milking directly from farmers, 21 samples were from collections points after collecting from all suppliers, and 4 milk samples were from dairy collection ramp after mixing milk from all the production lines.

Milk samples were taken in sterile plates of 100 ml, cooled and transported to the laboratory.

The number of microorganisms (TNM) was determined after inoculating a nutrient culture medium and incubating for 72 h at 30°C, and was expressed as the logarithm of the number of microorganisms per ml of milk. The number of bacilli (Nbac) was determined after inoculating a nutrient culture medium after warming the milk for 10 min. at 80°C, and was expressed as the logarithm of the number per ml.

Bacillus ability to grow in psychrotrophic conditions was examined by keeping the thermally treated milk for 10 days at the temperature of 7°C, and Bbac was determined as in the previous procedure. The biochemical activity of isolated bacilli was determined from the ability of bacilli to grow at various temperatures (45 and 55°C), in the presence of 7% NaCl and at pH 5.7. Bacillus pathogenicity was determined from their ability to haemolyse blood agar or to degrade lecithin.

Enzyme activity of bacillus strains significant for production processes in the dairy industry such as hydrolysis of starch, gelatin and casein, was investigated in culture media with added starch, gelatin or casein after incubation for 24 h at 30°C.

Starch degradation activity was determined by the appearance of a light zone around colonies enhanced by lugol. Gelatin degradation was confirmed in the cases when the culture medium remained liquid after standing in the refrigerator for 4 hours. Casein hydrolysis could be seen in casein agar as the appearance of clear zone around bacillus colonies.

RESULTS AND DISCUSSION

Investigation of the contamination of fresh milk microorganisms, showed that the level of contamination directly depended on the place where samples had been taken.

Table 1. Microorganism numbers in fresh milk in relation to the place where milk samples had been taken

place	Average log value TNM and Nbac per ml of milk					
	TNM*			Nbac**		
	n	$\bar{X} \pm S$	CV%	n	$\bar{X} \pm S$	CV%
individual farms	89	4.84 ± 0.39	8	89	0.92 ± 0.81	88
purchase point	21	5.43 ± 1.07	20	21	1.56 ± 0.66	42
collection point	4	5.83 ± 0.53	9	4	1.56 ± 0.42	27

*Total Number of Microorganisms/ml of milk

**Number of bacilli / ml of milk

The results presented at Table 1 show that the contamination of milk samples at collective places was an order of magnitude greater than in individual milk samples. The presence of mesophyl bacilli in fresh milk ranged from 0.92 ± 0.81 log to 1.56 ± 0.42 log depending on the place where the milk samples had been taken. The coefficient of variation (Cv) of the total number of microorganisms showed that the extent of overall milk contamination with microorganisms was similar at the places where the samples had been taken. However, the presence of bacilli in milk was very variable and the greatest differences could be noticed between individual milk samples. This may be related to the hygienic conditions in the milking procedure.

After thermal processing (80°C , 10 min) and determining the initial number of mesophyl bacilli, the milk was kept for 10 days at the temperature of 7°C , which enabled the growth of psychrotrophic bacilli. The results are presented in Table 2.

Table 2. Bacillus growth during storage of thermally processed milk under psychrotrophic conditions

Groups	Average log value of bacilli per ml of milk					
	1 st day			10 th day		
	n	$\bar{X} \pm S$	CV%	n	$\bar{X} \pm S$	CV%
first	35	1.57 ± 0.61	38	35	0.72 ± 0.60	83
second	17	1.07 ± 0.87	81	17	0.0	0
third	15	0.54 ± 0.73	130	15	0.54 ± 0.76	130
fourth	41	0.81 ± 0.78	96	41	1.73 ± 0.86	49
fifth	6	0.0	0	6	2.07 ± 0.47	22

According to increases or decreases in the numbers of bacilli during storage at 7°C , 114 samples of milk were divided into five groups. In the first group it was noticed that the average log of bacillus number decreased during storage in psychrotrophic condition. In the second group the number fell very steeply to less than 1/ml of milk - In fifteen milk samples (third group) the number did not change between the first and tenth day. In the fourth group, bacillus number

increased after keeping the milk in psychrotrophic conditions and in the fifth group bacillus number on the first day was lower than 1 bacillus/ml of milk, but after 10 days there was a significant increase to a mean value of 2.07 log. The results indicate the presence of psychrotrophic bacilli which can multiply at 7°C, and in that way spoil the milk. The results also raise the suspicion that these bacilli may represent a risk for human health.

The differences between mean values on the first and tenth days of incubation in psychrotrophic conditions were subjected to the t-test (Table 3).

Table 3. t-test of differences between mean bacillus number on the first and tenth day of storage of groups of milk samples in psychrotrophic conditions

	milk samples grouped according to bacillus behaviour				
	first	second	third	fourth	fifth
1 st/10th day	5.91*	5.07*	1.27	5.02*	10.62**

*P < 0.01

**P < 0.001

A highly significant difference was found for milk samples in the first and second group, which showed that these bacilli do not grow in psychrotrophic conditions. The fourth and fifth groups showed contrary results confirming data obtained by other authors (Giffel et al., 1996; Garcia-Armesto et al., 1994; Meer et al., 1993) that certain bacillus strains can grow in psychrotrophic conditions of storage.

The biochemical characteristics of bacilli isolated from the milk samples, important for the production process, are presented in Table 4.

Table 4. Biochemical characteristics of isolated bacillus strains important for the production process

Number of strains	growth at 45°C		growth at 55°C		growth in the presence of 7% NaCl		growth in broth at pH 5.7	
	N°	%	N°	%	N°	%	N°	%
135	133	98.51	121	89.62	125	92.59	132	97.77

Most isolated bacillus strains grew at the temperature of 44 and 55°C, and they were not inhibited by the concentration of 7% NaCl, nor by the environmental pH of 5.7. The ability of bacilli to grow in conditions of high temperatures, increased salt quantity or low pH, confirms that these microorganisms survived production processes and operations that are usually undertaken in food production. Certain bacillus strains have been characterized as causes of poisoning in people (Garcia et al., 1994; Molin, 1994). Among the biochemical characteristics of bacilli, it was noticed that bacilli which have the ability to haemolyse blood agar and degrade lecithin could be pathogenic for people. Our results for thermally treated milk during storage for 10 days at 7°C are presented in Table 5.

Table 5. Presence of lecithin positive and haemolytic strains of bacilli

charact.	first day			tenth day at 7°C			total	
	number of strains	positive		number of strains	positive		number of strains	positive
		N°	%		N°	%		N°
haemolysis	108	47	43.51	27	13	48.15	135	60
lecith. pos.	108	71	65.74	27	20	74.07	135	91

Among 108 bacillus strains isolated from milk on the first day 43.51% were haemolytic and 65.74% degraded lecithin. The proportions were similar in same milk samples that had been kept for ten days at the temperature of 7°C (48.15% haemolytic and 74.07% lecithin positive).

The biochemical characteristics of bacilli isolated from the milk on the first and tenth day which were significant for production processes in the dairy industry are presented in Table 6.

Table 6. Enzyme activity of bacillus strains significant for production processes in the dairy industry

hydrolysis of	first day			tenth day at 7°C			total	
	number of strains	positive		number of strains	positive		number of strains	positive
		N°	%		N°	%		N°
starch	108	43	39.81	27	11	40.74	135	54
gelatin	108	8	7.41	27	0	0	135	8
casein	108	86	79.63	27	23	85.18	135	109

Most bacillus strains degraded casein. Their ability to break down casein points to the increased danger of sweet milk curdling during storage or of the appearance of bitter taste from bitter peptides produced from beta casein hydrolysis (Babic et al., 1985; Puhon, 1996). Starch degradation by bacillus enzymes was similar on the first and tenth day of incubation in psychrotrophic conditions, and it is an undesirable characteristic of bacilli because starch is used in the dairy industry as a stabilizer.

CONCLUSION

Examination of 114 milk samples for the presence of bacilli, showed that bacilli occurred at different levels depending on the place where the samples were taken. Mean bacillus number in milk samples on the farm after milking was 0.92 ± 0.81 log/ml. In milk samples at the collection point, the mean bacillus number had increased to 1.56 ± 0.66 , and remained at 1.56 ± 0.42 log/ml in big dairies.

The number of bacilli in 114 milk samples was determined immediately the first day after treatment for 10 min. at 80°C, and after keeping that milk for 10 days

at 7°C. It was found that some bacilli grew in psychotrophic conditions. By t-test it was confirmed that the bacilli numbers increased or decreased significantly in different samples of milk.

A high percentage of isolated bacilli multiplied at the temperature of 45°C (98.51%) and 55°C (89.62%), in the presence of 7% NaCl (92.59%) and in broth at pH 5.7 (97.77%). It was also found that 48.15% of isolated strains were haemolytic and 67.40% degraded lecithin, which indicates that the isolated strains are possibly pathogenic.

Determination of the enzyme activity of isolated strains, showed that 80.74% degraded casein, 40.00% hydrolysed starch and 5.92% broke down gelatin. This enzyme activity of bacillus strains is significant for producing processes in the dairy industry.

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ISPITIVANJE BIOHEMIJSKIH OSOBINA BACILA IZOLOVANIH IZ SIROVOG MLEKA

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SADRŽAJ

Pregledano je 114 uzoraka sirovog mleka na prisustvo bacila. Utvrđeno je da nalaz bacila u sirovom mleku zavisi od mesta uzorkovanja. Sirovo mleko pojedinih proizvođača kontaminirano je bacilima na nivou 0,92 log, na otkupnom mestu mleko sadrži bacile na nivou 1.56 log a u sabirnoj mlekari nivo bacila je takođe 1.56 log/ml. Izolacija bacila iz mleka sledila je posle zagrevanja mleka 10 min na 80°C, zasejavanjem na hranjivu pologu i inkubacijom 48-72 časa na 30°C. Sposobnosti bacila da rastu u psihrotrofnim uslovima određeno je čuvanjem termički obrađenih uzoraka mleka 10 dana na temperaturi 7°C. Razmnožavanje bacila u psihrotrofnim uslovima je veoma različito i uzorci mleka se mogu podeliti u pet grupa. U prvoj grupi zapažamo da se broj bacila u psihrotrofnim uslovima smanjio (log. 1.57 na log 0.72) što predstavlja visoko značajnu razliku sredina određenu t testom. U drugoj grupi su bacili koji ne rastu i ne preživljavaju u uslovima niskih temperatura i posle 10 dana čuvanja uzoraka mleka na 10°C broj

bacila je manji od 1/ml mleka. Treća grupa bacila ne pokazuje razlike u broju bacila pre i posle čuvanja uzoraka mleka u psihotrofnim uslovima. Četvrta grupa bacila se razmnožava u uzorcima mleka u psihotrofnim uslovima i daje razliku sredina izraženu t testom kao visoko značajnu. Peta grupa bacila pripada psihotrofnim sojevima jer se dokazuje u psihotrofnim uslovima čuvanja mleka.

Ispitivanje sposobnosti rasta bacila u zavisnosti od karakteristika medija, utvrđeno je da od 135 izolovanih sojeva 133 rastu u bujonu na temperaturi 45°C, 121 soj raste na 55°C 125 sojeva bacila rastu u prisustvu 7% NaCl a čak 132 soja rastu u bujonu sa pH 5.7. Više od polovine ispitivanih sojeva (67.40%) je lecitin pozitivna i 48,15% sojeva je hemolitično što ukazuje na moguće prisustvo bacila patogenih za ljude. Enzimska aktivnost izolovanih sojeva bacila značajnih za tehnološke procese u mlekarstvu ukazuje da 40,0% razlaže skrob, 5,92 želatin i da 80,74% sojeva hidrolizuju kazein.