The survival of *Streptococcus thermophilus*, *Lactobacillus acidophilus* and *Bifidobacterium* spp. during the storage of fermented milk

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Summary

The viable counts of Lactobacillus acidophilus (L. acidophilus), Bifidobacterium ssp. and Streptococcus salivarius subsp. thermophilus (Str. thermophilus) and the survival of these bacteria in commercial fermented milks during the four weeks storage at 7 ± 1 °C were investigated. The titratable acidity and pH value of samples were measured as well. The fermented milk samples in original packaging were supplied by three dairies (coded A, B and C) one-day post production. First analysis of samples were done 24 hours after manufacture. During storage, samples were tested in seven days intervals. Str. thermophilus populations were in the range of 3×10^8 to 9×10^8 cfu/g and survived at levels greater than 10^8 cfu/g till the end of the storage for all fermented milk samples. The highest counts of L. acidophilus were found in samples A, but also in samples B while in C remained above the recommended limit of one million viable cells per gram throughout the storage period. The most variable was the count of Bifidobacterium spp. Mean values for the viable count one day post-production ranged from 4.6×10^2 to 2.3×10^6/g.

Key words: fermented milk, storage, Lactobacillus acidophilus, Streptococcus salivarius subsp. thermophilus, Bifidobacterium spp.

Introduction

From the historical perspective, fermentation of milk with lactic acid bacteria not only contributed in variety of milk products but also resulted in foods with improved shelf life. New, in this context, are fermented milk products with special characteristics or functions either "protective" or "therapeutic".

Lactic acid bacteria (LAB) are normal residents of the gastrointestinal tract of human beings and it is agreed that their presence in the gastro-intestinal tract is essential for healthy life. The viable bacteria that influence the health of the host in a beneficial manner are called probiotics (Fuller, 1992). Probiotic bacteria are nowadays used to treat disturbed intestinal microflora which may lead to diarrhoea, mucosal inflammation or activation of harmful drugs and carcinogens in intestinal contents (Siitonen et al., 1990, Isolauri et al., 1991, Salminen and Isolauri, 1994, Salminen et. al., 1996). Several basic properties are required for an effective probiotic strain of LAB. Among the most important properties is the ability to survive and grow in the intestinal tract. Although yoghurt bacteria (*L.delbrueckii* subsp.*bulgaricus* and *Str. salivarius* subsp. *thermophilus*) have been found to be beneficial for human health and nutrition they are not typical members of the intestinal flora of men and the ability to colonise the gut is extremely doubtful (F u l l e r, 1991). Cultures most often mentioned as probiotics for humans include *Lactobacillus acidophilus, Lactobacilus casei* and *Bifidobacterium* species (G i l l i l a n d, 1998). The probiotic strains used are mostly intestinal isolates.

Industrial production of fermented milks with probiotic bacteria of intestinal origin initially encountered several problems: human strains usually grew slowly in milk, and tended to lose viability during storage, flavour and aroma were lacking. To avoid these problems probiotic bacteria are used in mixed starters most often with yoghurt bacteria (M a r s h a l l, 1991).

To convey many of the claimed health benefits attributed to probiotic organisms the ingestion of live organisms is necessary (L e e and S a l m i n e n, 1995, M a r s h a l l, 1996). Suggested minimum level for probiotic bacteria in yoghurt or other fermented milk is 10^6 viable cells per ml or g of product (K u r m a n, 1988, K u r m a n and R a š i ć, 1991).

The aim of this study was to determine the viable count of *Lactobacillus acidophilus* (*L. acidophilus*), *Bifidobacterium* spp. and *Streptococcus salivarius* subsp. *thermophilus* (*Str. thermophilus*) in three commercial fermented milks and to investigate the survival of this bacteria during the four weeks storage at 7 ± 1 °C.

Materials and methods

Sampling and testing protocol

The fermented milk samples in original packaging were supplied by three dairies one-day post production with the temperature on receipt being at or below 5 °C. First analysis were done 24 hours after manufacture. Samples were stored during four weeks at 7 ± 1 °C in refrigerator and every seven days new original packing samples were taken from refrigerator for analysis. From the dairies coded A and B samples for experimental purpose were taken 7-times and from the dairy C 3-times. All analyses were done in duplicate.

Fermented milk

Starter cultures used for production of fermented milk coded A and C were composed of *L. acidophilus, Bifidobacterium* spp. and *Str. thermophilus*. Products A and C were supplied in 0,5 l PURE-PACK packaging. Fermented milk coded B was produced with mixed starter culture of *L. acidophilus, Bifidobacterium* spp., *Str. thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* (*L. bulgaricus*) and packed in 150 g cups.

Media

Maltose MRS (M-MRS) agar was used for enumeration of *L. acidophilus*. M-MRS agar was prepared by mixing all components of MRS agar except glucose which was substituted with an equal amount of maltose. Maltose was dissolved in distilled water, filter sterilised and added to the autoclaved base medium. Viable cells of *Bifidobacterium* spp. were enumerated using MRS agar supplemented with 5 % of NNLP. NNLP was prepared as follows: 6.00 g LiCl (Merck), 0.03 g nalidixic acid (Sigma N-8878), 0.20 g neomycin-sulphate (Sigma N-1976) and 0.25 g paromomycin sulphate (Parke-Davis) were suspended and adjusted to 100 ml with distilled water; pH was adjusted with 0.1 M NaOH to 7.2-7.5, solution was filter sterilised and added to MRS before use. The M17 agar (Merck 1.15029) was used for enumerating *Str. thermophilus.*

Enumeration of lactic acid bacteria

One gram of sample was diluted with 9 ml of Ringer solution (M e r c k, 1.15525) and mixed with a vortex mixer. After blending the sample was serially diluted in Ringer solution and dilutions were then plated in duplicate on the appropriate media using pour plate technique. Dilutions used were 10^{-3}, 10^{-4} and 10^{-5} for M-MRS, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} for MRS+NNLP and 10^{-4}, 10^{-5} and 10^{-6} for M17 agar.

Incubation was carried out at 37 °C for 72 h microaerobically (Generbag, bioMérieux) for M-MRS, at 37 °C for 72 h anaerobically (Generbag, bioMérieux) for MRS+NNLP and at 42 °C for 48 h aerobically for M17 media. After incubation plates with 30 to 300 colonies were counted.

Analysis

The pH values of the fermented milk samples were measured at 18 - 20 °C using "Iskra-MA 5722" pH meter calibrated with pH 4.0 and 7.0 standard buffers. The titratable acidity was determined according to Soxhlet-Henkel method (Methodenbuch VI).

Results and discussion

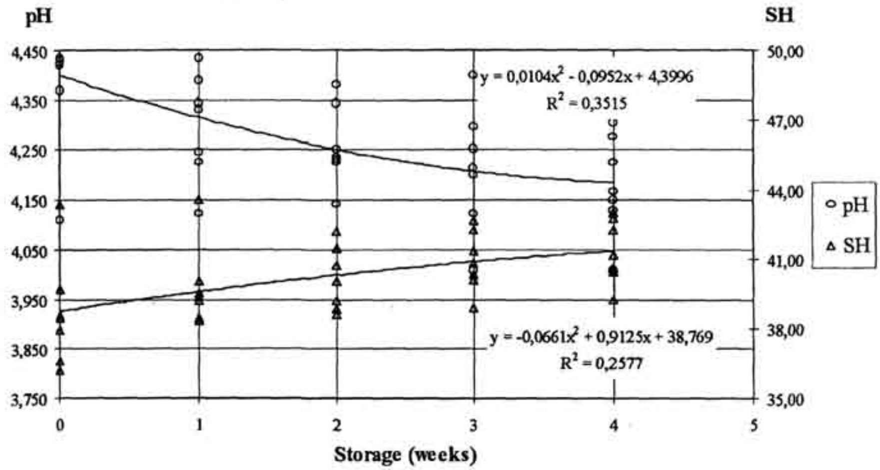
Titratable acidity and pH

The changes in titratable acidity (TA) and pH in fermented milks (A, B and C) during storage are illustrated in Figs. 1-3. The mean values (7 experiments in duplicate in the case of A and B and 3 experiments in duplicate in the case of C) for TA 24 h after production of fermented milks were 38.70 °SH, 40.81 °SH and 39.60 °SH, for A. B and C respectively. The increase of TA during the storage was minimal for the samples B and C (1.14 and 0.98 °SH respectively) and modest for the sample A (2.67 °SH). The mean values after four weeks were 41.37, 41.95 and 40.58, for A, B and C respectively. Practically the same overall increase in TA during 35 days of storage (at 4 °C) of fermented milks produced with mixed starter cultures of yoghurt and probiotic bacteria was found by D a v e and S h a h, (1997).

The mean pH values of samples A, B and C one-day post production were 4.40, 4.28 and 4.42, respectively. A gradual decrease in the pH was observed throughout the storage period of 4 weeks when the pH dropped to 4.18 for samples A, to 4.17 for samples B and to 4.13 for samples C. As shown in Figs. 1-3, the decrease was the least pronounced for samples B whose pH values were after production on average the lowest. Otherwise, the variability between experiments was quite pronounced. Anyway the results are well comparable with the results obtained by D a v e and S h a h, (1997) and M i c a n e l et al. (1997) working with the fermented milks of similar composition.

Figure 1: Changes in the titratable acidity (°SH) and pH during storage of fermented milk A

Slika 1: Promjene titracijske kiselosti (°SH) i pH vrijednosti tijekom skladištenja fermentiranog mlijeka A



Changes in bacteria population during storage

A change in the viable counts of *L. acidophilus, Bifidobacterium* spp. and *Str. thermophilus* during storage of fermented milks A, B and C is presented in table 1.

The only organism, which consistently yielded similar results in all experiments with fermented milk samples, was *Str. thermophilus*, which survived at levels greater than 10^8 cfu/g during the four weeks of storage. The viable counts of this organism were also the least variable which can be seen from the values of CV. These results are in agreement with the results obtained by others (D a v e and S h a h, 1997, M i c a n e l et al., 1997, R y b k a and K a i l a s a-

Table 1. Viable counts (in $\log_{10}$/g) of L. acidophilus, Bifidobacterium spp. and Str. thermophilus during storage of fermented milks A, B and C

Tablica 1. Broj živih stanica (u $\log_{10}$/g) of L. acidophilus, Bifidobacterium spp. i Str. thermophilus tijekom skladištenja A, B i C fermentiranih uzoraka mlijeka

Week* Tjedan*	n[a]		*Str. thermophilus* $\log_{10}$/g	CV (%)[c]	*L. acidophilus* $\log_{10}$/g	CV (%)	*Bifidobacterium* spp. $\log_{10}$/g	CV (%)
Fermented milk A Fermentirano mlijeko A								
0	14		8.71	1.84	7.97	2.58	3.25	10.77
1	14		8.77	1.18	7.94	3.08	3.15	10.39
2	14		8.71	0.71	7.82	2.52	2.89	15.41
3	14		8.63	1.87	7.81	1.91	2.79	12.94
4	14		8.57	2.63	7.66	2.66	2.77	12.01
Fermented milk B[b] Fermentirano mlijeko B								
0	14	(6)	8.48	3.51	7.93	2.15	2.67	19.35
		(8)					6.37	1.47
1	14	(6)	8.54	2.62	7.77	2.48	2.63	18.73
		(8)					6.31	1.09
2	14	(6)	8.40	2.12	7.45	4.38	2.63	18.73
		(8)					6.29	1.19
3	14	(6)	8.34	1.21	7.23	4.93	2.55	17.84
		(8)					6.21	1.79
4	14	(6)	8.03	6.24	6.80	7.41	2.55	17.84
		(8)					6.13	1.75
Fermented milk C Fermentirano mlijeko C								
0	6		8.97	0.84	7.10	1.69	5.46	1.29
1	6		8.98	0.80	7.08	0.51	5.42	1.55
2	6		8.97	1.21	6.96	0.91	5.34	2.07
3	6		8.92	1.05	6.82	3.27	5.28	2.61
4	6		8.83	1.12	6.73	1.45	5.18	1.83

* 0 = One day after production; 1, 2, 3, 4 = after 1, 2, 3 and 4 week of storage

* 0 = jedan dan nakon proizvodnje; 1,2,3, 4, = nakon 1, 2, 3 i 4 tjedna skladištenja

[a] number of samples (experiments in duplicate); for example in the case of samples A - 7 experiments in duplicate

[a] broj uzoraka (paralelke); npr. u slučaju uzoraka A-7 eksperimenti su provedeni sa dvije paralelke

[b] the counts for *Bifidobacterium* spp. are grouped in two groups because in the first three experiments the viable counts were very low compared to next four experiments;

[b] broj *Bifidobacterium* spp. grupiran je u dvije skupine budući da je u prva tri eksperimenta broj živih stanica bio vrlo nizak u usporedbi sa naredna četiri eksperimenta.

[c] coefficient of variability %

[c] koeficijent varijabilnosti %,

Figure 2: Changes in the titratable acidity (°SH) and pH during storage of fermented milk B

Slika 2: Promjene titracijske kiselosti (°SH) i pH tijekom skladištenja fermentiranog mlijeka B.

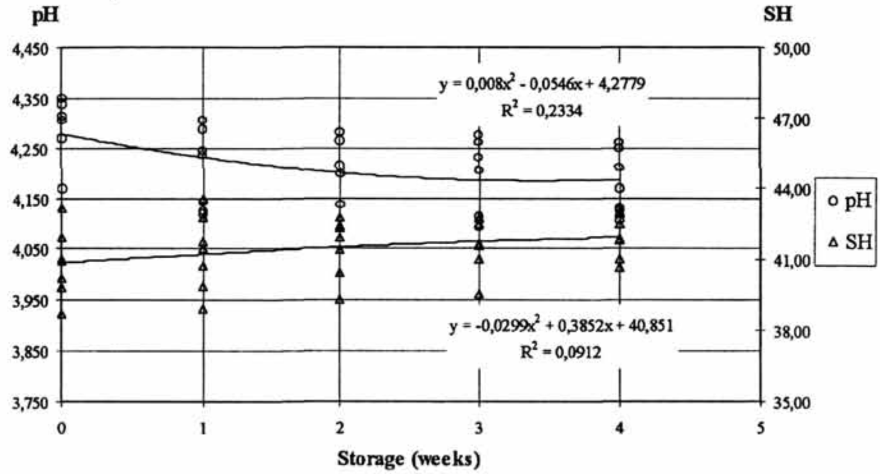
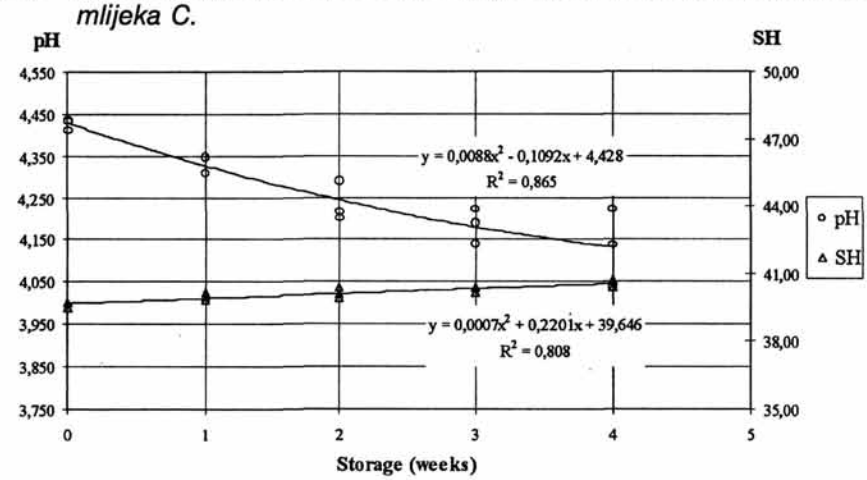


Figure 3: Changes in the titratable acidity (°SH) and pH during storage of fermented milk C

Slika 3: Promjene titracijske kiselosti (°SH) i pH tijekom skladištenja fermentiranog mlijeka C.



p a t h y, 1995) except that in some cases the counts were even greater. The highest counts of *L. acidophilus* were found in samples A, but also in samples B while in C remained within the recommended limit of one million viable cells per gram throughout the storage period. The CV were in limits of 0.51 to 4.93 % (except in one case) indicating acceptable variability between samples. R y b k a and K a i l a s a p a t h y (1995) and D a v e and S h a h (1997) observed less viability of *L. acidophilus* in yoghurts with *L. bulgaricus* which was not the case in our experiment. The most variable was the count of *Bifidobacterium* spp. As can be seen from the Table.1 that survival of these bacteria during storage was good, but the viable counts after fermentation were in most cases very low and under the recommended value of one million. Mean values for the viable count one-day post production ranged from 4.6 x 10^2 to 2.3 x 10^6/g. Lankaputhra et al. (1996) reported that any drop in pH below 4.3 greatly affected the viability of bifidobacteria. In our experiment, the pH values after production of fermented A and C milks were above this values, and the pH values of fermented milk B were in first six experiments around 4.3 and in seventh 4.17. The viable count of bifidobacteria, in this experiment, one-day post manufacture was 1.9 x 10^6. The greatest influence of pH on the viable count of bifidobacteria compared with *L. acidophilus* and *Str. thermophilus* can be seen from Figs. 4-6 where regressions of viable count of bacteria on pH are presented. The highest correlation coefficients were found for bifidobacteria in fermented milk B. Dave and Shah (1997) observed that associative yoghurt bacteria affected the multiplication of bifidobacteria during manufacture of yoghurt. Furthermore, the same authors concluded that variations in microbial counts were most likely due to the changes in oxygen content, because other factors such as TA, pH and hydrogen peroxide remained almost unchanged during storage.

Figure 4: Regression of viable count of lactic acid bacteria on pH value for fermented milk A (viable count is expressed as log_{10}/g)

Slika 4: Regresija broja živih stanica mliječno-kiselih bakterija o pH vrijednosti fermentiranog A mlijeka (broj živih stanica izražen je kao log_{10}/g)

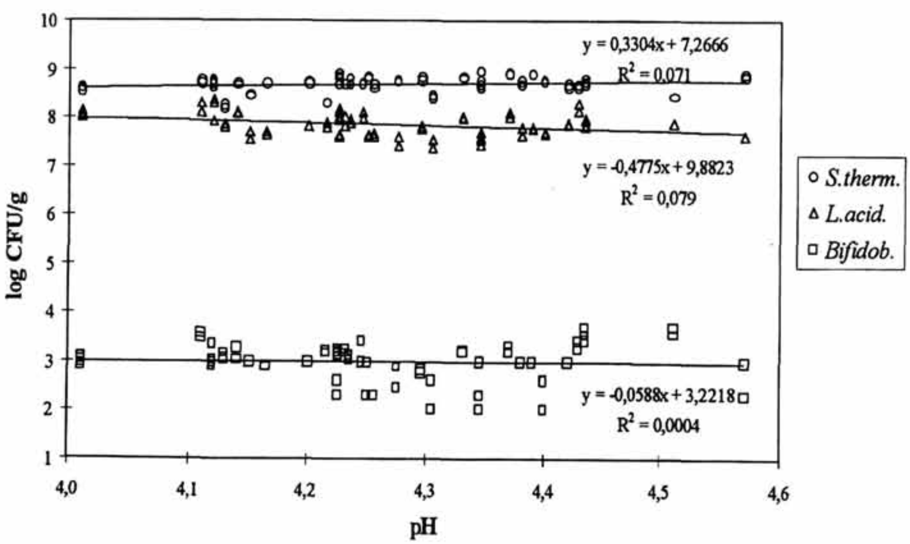


Figure 5: Regression of viable count of lactic acid bacteria on pH value for fermented milk B (viable count is expressed as log_{10}/g)

Slika 5: Regresija broja živih stanica mliječno-kiselih bakterija o pH vrijednosti fermentiranog B mlijeka (broj živih stanica izražen je kao log_{10}/g)

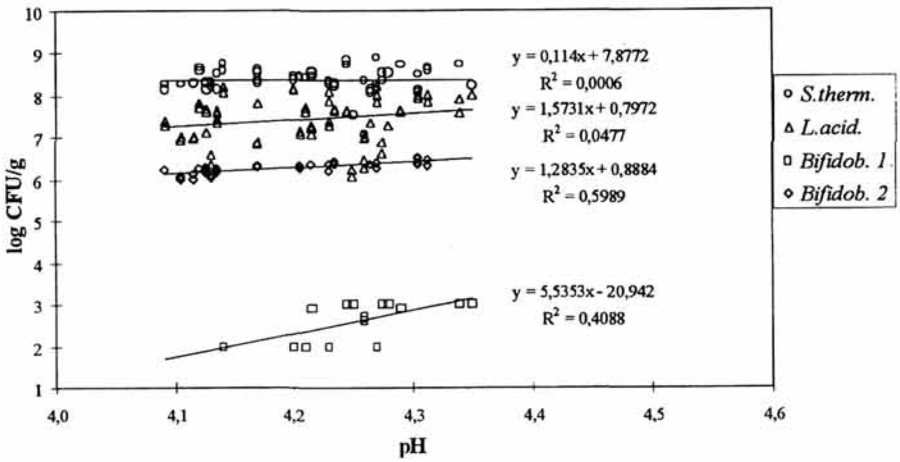
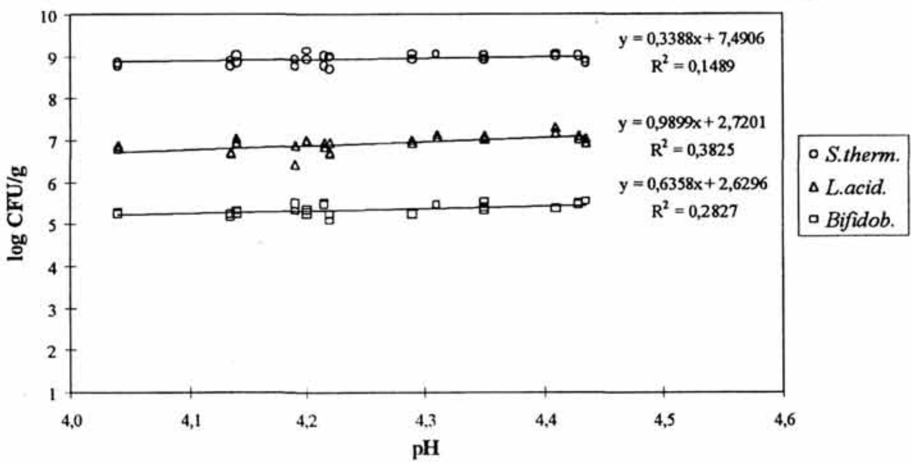


Figure 6: Regression of viable count of lactic acid bacteria on pH value for fermented milk C (viable count is expressed as log_{10}/g)

Slika 6: Regresija broja živih stanica mliječno-kiselih bakterija o pH vrijednosti fermentiranog C mlijeka (broj živih stanica izražen je kao log_{10}/g)



Conclusions

The fermented milks tested showed similar patterns of increase or decrease in titratable acidity and pH during the storage for four weeks at 7 ± 1 °C. The viabilities of *Str. thermophilus, L. acidophilus* and *Bifidobacterium* spp. during storage were comparable as well. However, there were the notable differences in the viable count of these bacteria one-day post manufacture of fermented milks. *Str. thermophilus* populations were in the range from 3 x 10^8 to 9 x 10^8 cfu/g, *L. acidophilus* from 10^7 to 9 x 10^7 cfu/g and *Bifidobacterium* spp. from 4 x 10^2 to 2 x 10^6 cfu/g. *Str. thermophilus* was the dominant species in all fermented milks during the entire period of storage. The highest counts of *L. acidophilus* were found in samples A, but also in samples B while in C remained within the recommended limit of one million viable cells per gram throughout the storage period. The population of bifidobacteria is the most variable but to our opinion the secondary problem is not in surviving of well-selected bifidobacteria in mixed starter culture but in carrying out the correct fermentation and manufacture processes.

PREŽIVLJAVANJE STREPTOCOCCUS THERMOPHILUS, LACTOBACILLUS ACIDOPHILUS I BIFIDOBACTERIUM SPP. TIJEKOM SKLADIŠTENJA FERMENTIRANOG MLIJEKA

Sažetak

Određivan je broj Lactobacillus acidophilus (L. acidophilus), Bifidobacterium ssp. i Streptococcus salivarius subsp. thermophilus (Str. thermophilus) u komercijalnim fermentiranim uzorcima mlijeka te preživljavanje navedenih bakterija tijekom skladištenja kroz 4 tjedna pri temperaturi od 7± 1 °C. Također je provedeno i određivanje titracijske kiselosti i pH vrijednosti uzoraka. Fermentirani uzorci mlijeka u originalnoj ambalaži dobavljeni su iz tri mljekare (označene sa A, B i C) nakon prvog dana proizvodnje. Prva analiza provedena je nakon 24 sata od završetka proizvodnje. Tijekom skladištenja uzorci su analizirani u vremenskom intervalu od 7 dana. Populacija Str. thermophilus iznosila je od 3 x 10^8 do 9 x 10^8 cfu/g, a nivo preživljavanja na kraju skladištenja bio je veći od 10^8 cfu/g, za sve fermentirane uzorke mlijeka. Najveći broj L. acidophilus, tijekom skladištenja, određen je u uzorcima A, zatim u B dok se u C zadržao iznad preporučene vrijednosti od jedan milijun živih stanica po gramu. Najveće promjene uočene su u broju Bifidobacterium spp. Tako je srednja vrijednost živih stanica, nakon provog dana proizvodnje, iznosila od 4.6 x 10^2 do 2.3 x 10^6/g.

Ključne riječi: fermentirano mlijeko, skladištenje, Lactobacillus acidophilus, Streptococcus salivarius subsp. thermophilus, Bifidobacterium spp.

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