



# Fermentation of Cow's Milk and Soy Milk Mixture with *L. acidophilus* Probiotic Bacteria with Yoghurt Culture

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**Abstract.** The aim of the paper was to produce a functional product and to determine the physico-chemical, microbiological and sensory properties of probiotic beverages produced from different mixtures of cow's milk and soy milk. The ratios of cow's milk and soy milk were 100:0%, 25:75%, 50:50%, 75:25% and 0:100%. The samples were fermented with probiotic bacteria *Lactobacillus acidophilus* (La5) with the addition of yogurt culture. The fermentation of samples was performed at +43 °C until coagulation and pH value of 4.6. Characteristics of the obtained beverages were monitored during storage at 1st, 7th, 14th and 21st day of storage at +4 °C. Produced probiotic beverages were observed and change in active acidity, titration acidity, change in the number of probiotic bacteria, and sensory properties and acceptability of products were tracked. After fermentation, the number of lactobacilli in the samples produced gradually decreased during 21 days, but was still above the probiotic minimum (CFU 10<sup>6</sup> mL<sup>-1</sup>) so the samples had probiotic properties. Samples of fermented beverages got the best score of their sensory properties on the seventh day of preservation. The sensory properties of the samples were mainly influenced by the type and ratio of the used milk. Mixing cow's milk with soy milk significantly improved the sensory properties of the product. The acceptance test showed good acceptance of fermented beverages samples by potential consumers, apart from the sample that was 100% soy milk.

**Keywords:** Cow's milk · Soy milk · Probiotics · *Lactobacillus acidophilus*

## 1 Introduction

Soy milk contains various oligosaccharides, including raffinose and stachyose, which can lead to digestive disorders [1]. Fermentation of soy milk gives the ability to transform and improve the taste and texture [2, 3]. Also, fermentation of soy milk is

considered as a good basis for the functional food development and production. Fermentation of soy milk with probiotic bacteria reduces the level of oligosaccharide and raises the level of free isoflavones [4]. Fermentation of soy milk with probiotic bacteria has numerous health benefits, including antimicrobial, antimutagenic, anticarcinogenic, hypertensive properties, and it is also reducing serum cholesterol and mitigating lactose intolerance [5, 6]. For fermented probiotic products, the ability of culture to make the positive influence on the sensory properties of the product is essential. The aim of this study was to produce a functional milk beverage (probiotic yoghurt) that has useful characteristics of probiotic and soy milk. For this purpose, the physico-chemical, microbiological and sensory properties of fermented probiotic beverages produced from different ratios of cow's milk and soy milk were estimated using *L. acidophilus* probiotic bacteria during 21 days of storage.

## 2 Materials and Methods

Five different proportions of homogenized milk in soy beverage (100:0, 75:25, 50:50, 25:75, 0:100) were prepared by mixing UHT sterilised milk and soy milk. Samples were inoculated with probiotic starter culture (La5) and yoghurt culture (YF-L811). Probiotic monocultures for inoculation were in a lyophilized DVS (Direct Vat Set) form. After incubation, the inoculum was cleaved into milk samples intended for the production of probiotic beverages. Fermentation was carried out at 43 °C until pH reached a value of 4.6. Characteristics of the obtained probiotic dairy beverages are monitored during and at the end of fermentation. Three different repeated-batch fermentations were performed.

### 2.1 Microbiological Analysis

The viable cell counts of the probiotic strain in the produced samples were determined by a standard pour plating method using the MRS agar (Merck, Germany) supplemented by clindamycin (Sigma-Aldrich, Germany). Clindamycin was added to a sterilized MRS agar cooled to 43 °C just before pouring into the Petri dish in order to prevent the growth of the used yoghurt culture during the fermentation process (ISO/IDF (2006)). Subsequently, Petri dishes were incubated at 37 °C for 72 h after which colonies of probiotic bacteria (La5) were enumerated. The obtained data represent the arithmetic average of the enumerated colonies expressed as CFU mL<sup>-1</sup>.

### 2.2 Physical, Chemical and Sensory Analysis

The active acidity (pH) of probiotic beverages was determined by the pH meter pH3110 (Portable meter ProfiLine) and the titratable acidity by the Soxhlet - Hankel method and expressed as % of lactic acid. During the 21 days of produced probiotic fermented beverage samples storage, changes in acidity (pH value and lactic acid content) were observed, as well as the microbiological and sensory properties of the product. Sensory properties are rated by a weighted scoring method (ISO,1985) by a group of 5 trained sensory analysts. Eligibility of probiotic beverages was performed

by testing 30 younger consumers (students around 20 years old) using the verbal 9-point Hedonic Scale (Periam) [7].

### 2.3 Statistical Analysis of Results

The results of the analyzed samples are shown as the average repetition value ± standard deviation. One-way analysis of variance (ANOVA) and multiple comparisons (Duncan's post-hoc test) were used to estimate significant difference in data at the significance level of  $p < 0.05$ . Statistics were implemented using Microsoft Office 2014 and demo versions of the MS Office XLSTAT-Pro 2014 statistical package.

## 3 Results and Discussion

The fermentation of milk and soy milk samples (100:0, 0:100, 25:75, 50:50, 75:25) with probiotic bacteria La5 and the starter culture addition, lasted 5 to 7 h (Table 1). Table 1 shows changes in pH value during fermentation in all analyzed samples.

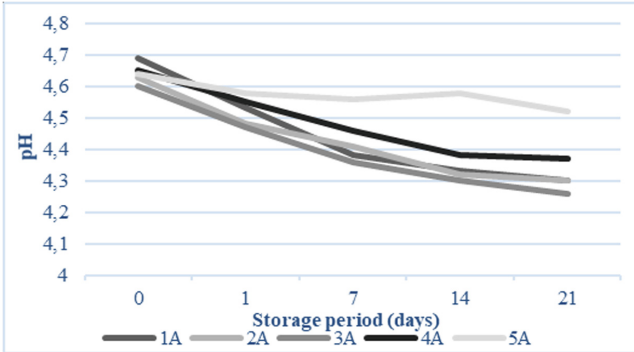
**Table 1.** Fermentation time and acidity of produced yoghurt samples

Para's	Yogurt samples				
	1A	2A	3A	4A	5A
Fermentation time (minutes)	420	360	360	360	360
pH	4.57 ± 0.08 <sup>ab</sup>	4.49 ± 0.05 <sup>bc</sup>	4.47 ± 0.02 <sup>c</sup>	4.57 ± 0.04 <sup>ab</sup>	4.59 ± 0.02 <sup>a</sup>
% lactic acid	0.67 ± 0.002 <sup>a</sup>	0.67 ± 0.007 <sup>a</sup>	0.58 ± 0.003 <sup>b</sup>	0.53 ± 0.01 <sup>c</sup>	0.47 ± 0.001 <sup>d</sup>

1A–100% cow's milk; 2A–75% cow's milk + 25% soy milk; 3A–50% cow's milk + 50% soy milk; 4A–25% cow's milk + 75% soy milk; 5A–100% soy milk; Data represent mean values (±SD) of three repetitions. <sup>abc</sup> Duncan's Test ( $p < 0.05$ ) confirmed the statistically significant difference between mean values (±SD).

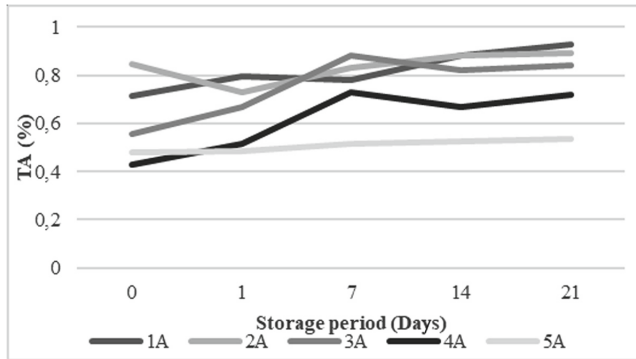
A decrease in pH value of Sample 1A (100% cow's milk) at the beginning of fermentation was rather slow, probably due to the presence of cow milk proteins with higher buffer capacity than that of soy protein [8, 9]. In general, it could be noticed that increasing soy milk share was accompanied by a decrease in pH and increased acidity, which could be attributed to the significantly reduced buffer capacity [10, 11]. This justifies the fact that the fermentation of sample 1 (100% cow's milk) lasted longer in relation to other samples with equal fermentation time. The inclusion of soy milk in cow's milk resulted in an increase in the rate of pH drop during fermentation as well as increase in acidity. Acidity analysis results of the samples during the storage days are shown in Figs. 1 and 2. During 21 days of storage at +4 °C, the dynamics of the various decreases in the pH value in the time sequence were observed. Namely, the decrease in pH values is caused by organic acids due to fermentation of carbohydrates with probiotic bacteria and starter cultures [8, 12]. The greatest decrease in pH value happened between day 1 and day 7 - with sample produced from 100% cow's milk (1A) and with samples produced from different ratios of cow's milk and soy milk, while in soy milk samples (5A) – very small decrease in pH values throughout the

storage period was observed. Also, in samples 4A, between day 14 and day 21 almost no pH change occurred. It was found that the titration acidity increase was proportional to the pH decrease (Fig. 2). Titration acidity is expressed as a percentage of lactic acid.



Legend: 1A-100% cow's milk; 2A-75% cow's milk + 25% soy milk; 3A-50% cow's milk + 50% soy milk; 4A-25% cow's milk + 75% soy milk ; 5A-100% soy milk . The data represents the mean of three repetitions.

**Fig. 1.** Change in pH values of cow’s milk and soy milk yoghurt fermented with probiotic bacteria La5 during the storage



Legend: 1A-100% cow's milk; 2A-75% cow's milk + 25% soy milk; 3A-50% cow's milk + 50% soy milk; 4A-25% cow's milk + 75% soy milk ; 5A-100% soy milk . The data represents the mean of three repetitions. The titration acidity (TA) is expressed as the percentage of lactic acid.

**Fig. 2.** Change of TA (%) during the storage of cow and soy milk yoghurt fermented with probiotic bacteria *Lactobacillus acidophilus*

The results of the change in the number of bacteria (La5) in the probiotic beverages of soy and cow’s milk during the storage are shown in Table 2. According to the results from Table 2, during 21 days of storage in the refrigerator in all samples, except for the

4A sample, the number of live lactobacillus cells increased. Although a significant decrease in pH values between the first and the seventh day occurred, this did not significantly affect the survival of lactobacilli. A sudden increase in acidity in yoghurt reduces the probiotic bacteria survival ability [13]. Mortazavian et al. [14], stated in their paper that the survival of lactobacilli in yoghurt is related to storage temperature, and the ideal temperature for storing this product is 2 °C. Lowering the temperature during the preservation of yoghurt reduces the activity of the yoghurt culture (*L. delbrueckii* subsp. *bulgaricus*) and increases the conditions suitable for growth of lactobacilli that have the ability to adapt.

**Table 2.** Change in the number of growing colonies (log CFU mL<sup>-1</sup>) *Lactobacillus acidophilus* (La5) during the cow's milk and soy milk fermented samples storage

Sample	Number of bacteria (log CFU/ml)				
	0	1	7	14	21
1A	8.29 ± 0.16 <sup>a</sup>	7.98 ± 1.13 <sup>a</sup>	8.19 ± 0.12 <sup>a</sup>	7.91 ± 1.14 <sup>ab</sup>	7.11 ± 0.83 <sup>b</sup>
2A	7.64 ± 0.20 <sup>a</sup>	7.52 ± 0.29 <sup>a</sup>	7.75 ± 0.35 <sup>a</sup>	8.22 ± 0.00 <sup>a</sup>	6.53 ± 0.00 <sup>b</sup>
3A	8.41 ± 0.83 <sup>a</sup>	7.97 ± 0.30 <sup>a</sup>	8.25 ± 1.05 <sup>a</sup>	6.79 ± 0.08 <sup>c</sup>	7.11 ± 0.00 <sup>b</sup>
4A	8.03 ± 0.07 <sup>a</sup>	8.2 ± 1.13 <sup>a</sup>	7.54 ± 0.79 <sup>a</sup>	7.02 ± 0.00 <sup>bc</sup>	n. d.
5A	7.97 ± 0.27 <sup>a</sup>	8.15 ± 1.12 <sup>a</sup>	7.29 ± 1.06 <sup>a</sup>	n. d.	8.49 ± 0.00 <sup>a</sup>

Legend: A - *L. acidophilus*; 1A–100% cow's milk; 2A–75% cow's milk + 25% soy milk; 3A–50% cow's milk + 50% soy milk; 4A–25% cow's milk + 75% soy milk; 5A–100% soy milk. Data represent mean values (±SD) of three repetitions. <sup>abc</sup> Duncan's Test (p < 0.05) confirmed the statistically significant difference between mean values (±SD).

Considering the results obtained, the best substrate for lactobacillus growth might be the 5A sample (100% soy milk) which is equal with the results from Farnworth et al. [8]. Presence of oligosaccharides that also function as prebiotics in soy beverage, contributes to lactobacillus growth. Also, the soy milk contains amino acids and peptides that promote the growth of probiotic bacteria [15]. During 21 days of storage, the least-increasing lactobacillus was found in sample 2A (75% cow's milk + 25% soy milk). According to Champagne and Gardner [16], La5 bacteria is growing better in soy milk than in cow's milk.

Sensory analysis was performed by a group of trained panelists who evaluated the scent, consistency, color, appearance and taste of samples (sample 1–5) of probiotic beverages during the 21 days of storage in the fridge at +4 °C. The evaluation results are shown in Table 3 – there is a clear comparison between the average scores of different samples (sample 1A-5A) of probiotic beverages produced by La5 and yoghurt culture.

**Table 3.** Sensory scores of probiotic beverages during storage.

Properties	Storage days	1A	2A	3A	4A	5A
Flavour (max 12)	1	10.5 ± 0.41 <sup>a</sup>	8.6 ± 2.35 <sup>b</sup>	8.08 ± 2.13 <sup>b</sup>	7.36 ± 2.75 <sup>bc</sup>	6.25 ± 2.78 <sup>c</sup>
	7	10.4 ± 1.08 <sup>a</sup>	9.3 ± 1.28 <sup>ab</sup>	8.89 ± 1.61 <sup>bc</sup>	8.17 ± 2.11 <sup>bc</sup>	7.67 ± 2.63 <sup>c</sup>
	14	9.4 ± 1.34 <sup>a</sup>	8.5 ± 1.41 <sup>ab</sup>	8.42 ± 1.71 <sup>ab</sup>	8.45 ± 1.82 <sup>ab</sup>	7.98 ± 2.29 <sup>b</sup>
	21	9.2 ± 1.19 <sup>a</sup>	8.0 ± 2.31 <sup>b</sup>	7.75 ± 2.91 <sup>c</sup>	7.75 ± 2.38 <sup>c</sup>	9.33 ± 1.91 <sup>a</sup>
Odour (max 2)	1	1.9 ± 0.18 <sup>a</sup>	1.52 ± 0.52 <sup>b</sup>	1.45 ± 0.59 <sup>b</sup>	1.38 ± 0.58 <sup>b</sup>	1.39 ± 0.72 <sup>b</sup>
	7	1.8 ± 0.39 <sup>a</sup>	1.74 ± 0.37 <sup>a</sup>	1.65 ± 0.47 <sup>a</sup>	1.68 ± 0.47 <sup>a</sup>	1.65 ± 0.56 <sup>a</sup>
	14	1.8 ± 0.30 <sup>a</sup>	1.70 ± 0.30 <sup>a</sup>	1.66 ± 0.38 <sup>a</sup>	1.65 ± 0.36 <sup>a</sup>	1.58 ± 0.43 <sup>a</sup>
	21	1.7 ± 0.43 <sup>a</sup>	1.61 ± 0.35 <sup>a</sup>	1.71 ± 0.32 <sup>a</sup>	1.69 ± 0.58 <sup>a</sup>	1.70 ± 0.38 <sup>a</sup>
Appearance (max 1)	1	0.9 ± 0.03 <sup>a</sup>	0.92 ± 0.18 <sup>a</sup>	0.90 ± 0.18 <sup>a</sup>	0.89 ± 0.19 <sup>a</sup>	0.85 ± 0.24 <sup>a</sup>
	7	0.9 ± 0.04 <sup>a</sup>	0.98 ± 0.04 <sup>a</sup>	0.92 ± 0.14 <sup>ab</sup>	0.88 ± 0.19 <sup>ab</sup>	0.83 ± 0.26 <sup>b</sup>
	14	0.9 ± 0.02 <sup>a</sup>	0.96 ± 0.07 <sup>ab</sup>	0.90 ± 0.21 <sup>ab</sup>	0.85 ± 0.20 <sup>b</sup>	0.87 ± 0.19 <sup>b</sup>
	21	0.9 ± 0.04 <sup>a</sup>	0.95 ± 0.09 <sup>a</sup>	0.89 ± 0.19 <sup>a</sup>	0.83 ± 0.29 <sup>a</sup>	0.91 ± 0.14 <sup>a</sup>
Color (max 1)	1	1.0 ± 0.00 <sup>a</sup>	0.90 ± 0.20 <sup>a</sup>	0.90 ± 0.17 <sup>a</sup>	0.85 ± 0.23 <sup>a</sup>	0.85 ± 0.24 <sup>a</sup>
	7	1.0 ± 0.00 <sup>a</sup>	0.97 ± 0.04 <sup>a</sup>	0.88 ± 0.15 <sup>ab</sup>	0.85 ± 0.18 <sup>b</sup>	0.83 ± 0.19 <sup>b</sup>
	14	1.0 ± 0.00 <sup>a</sup>	0.97 ± 0.05 <sup>a</sup>	0.92 ± 0.14 <sup>ab</sup>	0.88 ± 0.16 <sup>b</sup>	0.86 ± 0.17 <sup>b</sup>
	21	0.9 ± 0.08 <sup>a</sup>	0.96 ± 0.06 <sup>a</sup>	0.90 ± 0.16 <sup>ab</sup>	0.80 ± 0.31 <sup>b</sup>	0.94 ± 0.09 <sup>ab</sup>
Consistency (max 4)	1	<b>3.7 ± 0.40<sup>a</sup></b>	3.4 ± 0.67 <sup>ab</sup>	3.45 ± 0.60 <sup>ab</sup>	<b>3.29 ± 0.70<sup>ab</sup></b>	3.31 ± 0.73 <sup>b</sup>
	7	3.6 ± 0.53 <sup>a</sup>	3.5 ± 0.58 <sup>a</sup>	3.43 ± 0.91 <sup>a</sup>	3.30 ± 0.81 <sup>a</sup>	3.18 ± 0.98 <sup>a</sup>
	14	3.6 ± 0.41 <sup>a</sup>	3.5 ± 0.66 <sup>a</sup>	3.36 ± 0.92 <sup>a</sup>	3.24 ± 0.91 <sup>a</sup>	3.18 ± 0.78 <sup>a</sup>
	21	3.7 ± 0.34 <sup>a</sup>	3.03 ± 0.82 <sup>a</sup>	3.22 ± 0.38 <sup>a</sup>	<b>3.02 ± 1.18<sup>a</sup></b>	3.63 ± 0.47 <sup>a</sup>
Total (max 20)	1	18.2 <sup>I</sup> ± 1.7 <sup>a</sup>	15.3 <sup>II</sup> ± 3.6 <sup>b</sup>	14.6 <sup>III</sup> ± 03.3 <sup>b</sup>	13.6 <sup>III</sup> ± 3.8 <sup>b</sup>	12.7 <sup>IV</sup> ± 4.0 <sup>b</sup>
	7	18.0 <sup>I</sup> ± 1.5 <sup>a</sup>	16.7 <sup>II</sup> ± 1.6 <sup>ab</sup>	16.3 <sup>II</sup> ± 1.7 <sup>abc</sup>	15.30 <sup>II</sup> ± 2.5 <sup>bc</sup>	14.2 <sup>III</sup> ± 3.2 <sup>c</sup>
	14	16.8 <sup>II</sup> ± 1.6 <sup>a</sup>	15.8 <sup>II</sup> ± 1.8 <sup>ab</sup>	15.2 <sup>II</sup> ± 2.7 <sup>ab</sup>	15.02 <sup>III</sup> ± 3.0 <sup>ab</sup>	14.5 <sup>III</sup> ± 3.2 <sup>b</sup>
	21	<b>16.6<sup>II</sup> ± 1.6<sup>a</sup></b>	14.6 <sup>II</sup> ± 2.8 <sup>bc</sup>	14.4 <sup>III</sup> ± 2.4 <sup>c</sup>	<b>15.4<sup>II</sup> ± 1.7<sup>abc</sup></b>	16.4 <sup>II</sup> ± 1.9 <sup>ab</sup>

1A–100% cow’s milk; 2A–75% cow’s milk + 25% soy milk; 3A–50% cow’s milk + 50% soy milk; 4A–25% cow’s milk + 75% soy milk; 5A–100% soy milk. Data represent mean values (±SD) of three repetitions. <sup>abc</sup> Duncan’s Test (p < 0.05) confirmed the statistically significant difference between mean values (±SD).

After the first day of storage, the highest sensory scores were obtained with samples 1A (100% cow’s milk) and 2A (75% cow’s milk + 25% soy milk), while sample 5A (100% soy milk) was rated with the lowest scores. Such ratings clearly point to poorer sensory properties of soy milk, as the total grade of fermented beverage is almost proportional to the share of soy milk. However, seven days later, the ratings have changed significantly. In sample 1A the overall rating decreased, while in other samples - the overall rating increased. These changes are likely to be related to the acidity change, which negatively affects the taste of fermented cow’s milk – as proven by the results of other authors [17]. In sample 5A, the sensory taste rating increased with longer storage time. On the first day - it was 6.25, while on the 21st day 9.33, which contributed to an increase in the overall 5A sample rating. Also, in sample 5A (100% soy milk) there was a small decrease in pH value throughout the storage period, so acidification could not influence the evaluation. Although fermentation gives better flavor to a soy, beny taste is still present, proven by the lowest overall ratings, which is consistent with the results of the research conducted by Park et al. [18]. Sensory ratings of the consistency on the first day ranged from 3.29 to 3.74 compared to the highest

possible value 4. According to the results of the sensory analysis, it is evident that the best consistency ratings have samples 1 and 3.

Based on data determined by the hedonic scale, the basic statistical parameters ( $\bar{x}$ ,  $s$ ,  $C_v$ ) and desirability percentage are calculated (Table 4).

**Table 4.** Scores obtained of probiotic beverages after 1st day of storage at +4°C using hedonic scale

Scores	Samples of fermented beverages				
	1A	2A	3A	4A	5A
9	5	1	2	1	0
8	17	14	13	3	2
7	7	9	6	5	2
6	1	2	3	6	2
5	0	2	0	3	3
4	0	1	4	5	4
3	0	0	2	3	4
2	0	1	0	3	8
1	0	0	0	1	5
Total	30	30	30	30	30
X	7.9 <sup>a</sup>	7.1 <sup>ab</sup>	6.7 <sup>b</sup>	5.2 <sup>c</sup>	3.5 <sup>d</sup>
S	0.7	1.5	1.5	2.1	2.2
Desirability (%)	100 <sup>a</sup>	93 <sup>ab</sup>	75 <sup>b</sup>	33.3 <sup>c</sup>	13.3 <sup>d</sup>
Cv	9.3	20.7	22.7	40.7	61.2

1A–100% cow's milk; 2A–75% cow's milk + 25% soy milk; 3A–50% cow's milk + 50% soy milk; 4A–25% cow's milk + 75% soy milk; 5A–100% soy milk. Data represent mean values ( $\pm$ SD) of three repetitions.<sup>abc</sup> Duncan's Test ( $p < 0.05$ ) confirmed the statistically significant difference between mean values ( $\pm$ SD).  $\bar{x}$  = mean;  $s$  = standard deviation;  $C_v$  = variability coefficient.

The results are showing the opinion of potential customers (30) of new products. Samples 1A (100% cow's milk), 2A (75% cow's milk + 25% soy milk) and 3A (50% cow's milk + 50% soy milk) were rated as "I like it very much" by most of the respondents, using hedonic scale, which indicates that such type of products would be well accepted by consumers. Sample 4A (25% cow's milk + 75% soy milk) for most potential consumers was rated as acceptable, or as "I like it" on hedonic scale, meaning they would consume. From the other side, sample 5A (100% soy milk) received the lowest ratings. Most respondents rated it as "I do not like it" on the hedonic scale or "I extremely don't like it" and they would not consume. Similar research was conducted by Beherens et al. [19], for fermented soy beverage with the addition of various types of fruit and nuts.

## 4 Conclusions

Functional probiotic beverages were successfully produced from different ratios of cow's milk and soy milk mixture and the probiotic strain of *L. acidophilus* with yogurt culture. Adding soy milk to cow's milk resulted in probiotic beverages with high nutritional value. The number of probiotic bacteria in all samples of the produced beverages were in the range of 7.52–8.20 log CFU mL<sup>-1</sup>, which was above the generally accepted probiotic minimum (10<sup>6</sup> CFU mL<sup>-1</sup>), so the produced samples could be considered as probiotic products. Cow's and soy milk fermentation during the 21 days samples storage at +4 °C did not show a significant pH value decrease in all samples compared to typical pH value (pH = 4.5). During the 21 days of sample storage in the refrigerator at +4 °C, the number of lactobacilli gradually decreased, however, the number of lactobacilli live cells is above the probiotic minimum (10<sup>6</sup> CFU mL<sup>-1</sup>) so the samples could be considered as a probiotic product. The sensory properties of the samples were mainly influenced by the type and ratio of the milk used in the mixture. Mixing cow's milk with soy milk improved the sensory properties of the product, especially the odor, flavor and color. Samples of fermented beverages are best evaluated on the 7th day of storage, and with the further storage time, ratings became lower. The reason for bad rating is the increase in acidity, which is expressed with low sensory ratings of fermented beverage flavors. Acceptability Test has shown that the combination of cow's milk with soy milk up to 50% does not change the sensory properties of cow's milk, so this ratio of cow's milk and soy milk in fermented beverage production is acceptable to consumers. Such fermented products have high nutritional value and satisfy all requirements of functional foods, and could also be intended for different age groups of consumers.

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