Identification, characterization and industrial utilization of autochthonous strains of Streptococcus thermophilus isolated from Moldavian raw milk and dairy products of spontaneous fermentation

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Abstract

Introduction. Lactic acid bacteria with known metabolic properties contribute to the sensory characteristics of the products, to their quality and safety. The limited number of highly biotechnologically available strains and the constant risk of bacteriophage attacks justify the continued searching for new strains.

Materials and methods. Identification of thermophilic lactic acid bacteria was performant on the basis of phenotypic properties and using molecular methods – RAPD-PCR and FTIR spectroscopy. Antagonistic activity of selected strains was carried out by diffusion method. Starter culture and samples of yoghurt were manufactured according to the traditional technology.

Results and discussion. Selected strains of S. thermophilus are characterized by intense acidification of the milk for 3–4 hours, developing a milk acidity between 65–74 °T, forming a homogeneous, compact, dense coagulum, which ensures its firm consistency. The strains S. thermophilus strains CNMN-LB-50 and CNMN-LB-51 are capable of synthesizing exopolysaccharides (EPS).

The antagonistic activity against pathogenic microorganisms of strains of is high. The inhibition range varies between 16 and 18 mm from Escherichia coli. and 19-21 mm to Staphylococcus aureus, which inhibits the development of intestinal infections and prevents the development of pathogens in fermented milk samples. The maximum yeld of EPS synthesized at suboptimal temperature of 32 °C is 19.4% higher than at the optimal temperature for this process (40 °C) for S. thermophilus CNMN-LB-50 strain and 23, 8% for S. thermophilus CNMN-LB-51 strain. To stimulate the synthesis of EPS under industrial conditions in the manufacture of fermented milk products without altering the temperature, the nutrient medium should be supplemented with sucrose in an amount of 8%.

Conclusions. It is possible obtaining from the microflora of raw milk and dairy products of spontaneous fermentation the autochthonous strains of lactic bacteria with valuable biotechnological properties, intended for use in the composition of starter cultures for the production of fermented milk products.

Introduction

At the present, technological processes based on the activity of lactic acid bacteria (LAB) have a special significance. Modern dairy production is closely linked to the search and selection of suitable LAB with the specific properties, which implies an increase in the diversity of fermented dairy products. Thermophilic and mesophilic LAB are the most widely used as starter cultures for the production of fermented dairy products. The specific taste, consistency and other properties of the fermented dairy products depend on the strains which starter culture made up. The thermophilic LAB are capable to grow at temperatures of 37-45 °C that is an important condition associated with the technological process of manufacturing fermented dairy products such as yoghurt, baked fermented milk and various cheeses and play an important functional role in fermented dairy products [1].

Thermophilic cocci strains isolated from raw milk and spontaneous fermentation dairy products, in most cases, belong to Streptococcus and Enterococcus genera. Thermophilic LAB S. thermophilus are "useful" representatives for their application in starter cultures [2].

Identification process of an isolated strain includes its complete description and comparison with a reference strain. The rapid and specific classification of bacteria is an important task in microbiology, which can be achieved using traditional methods based on physiological, biochemical and molecular methods.

However, many scientists notice difficulties in differentiating and identifying of Streptococcus and Enterococcus bacteria using only the phenotype classification methods describing the inadequacy of the classical phenotype characteristics of these genes, such as the ability to grow on a medium containing esculin, bile and sugar fermentation, antibiotic resistance, and the range of typical growth temperature [3–5].

The aim of the present study was isolation and identification of S. thermophilus strains from raw milk and spontaneous fermentation dairy products and to study their industrial potential in order to create starter cultures which are suitable to yogurt production in local conditions.

Materials and methods

Reference bacterial strains

The following strains Streptococcus thermophilus A737 was obtained from Czech Collection of Microorganisms (Brno, Czech Republic), Streptococcus thermophilus 1241, Lactobacillus casei 4791were obtained from Collection of Microorganisms (Food Research Institute, Bratislava, Slovakia).

Isolation

10 grams of samples were homogenized with 90 ml sterile peptone physiological saline solution (5 g peptone, 8.5 g NaCl, up to 1000 ml distilled water, pH 7.0). The homogenate was diluted serially and the appropriate dilutions were surface plated on M17 agar media (Merck, Darmstadt, Germany), then plates were incubated at 37 °C for 72 hours.

Physiological and biochemical tests

Morphology. Pure cultures grown in skimmed milk in an incubator at 37 °C for 18 h was used. Before a microscope examination smears of cultures were stained with methylene blue for a few minutes.

Production of CO_2. 10 ml of M17 broth culture of the test strain incubated at 37 °C for 18 h covered the surface of the broth by molten overlay agar, precooled to 47 °C ± 1 °C, to a depth of 1 cm. Cultures were incubated for 1 week at 37 °C. The presence of gas is evident when the agar layer detaches itself from the underlying contents.

Catalase reaction. Mixed equal volumes of the M17 broth culture, incubated set at 37 °C for 18 h, with 150 $g \cdot l^{-1}$ hydrogen peroxide in a tube fitted with a rubber stopper. Gently turn the tube upside down once to favour mixing, and observe for bubbles of oxygen forming in the broth at room temperature over 20 min.

Action in litmus milk. In 10 ml of litmus milk (70 g litmus in 1000 mL skimmed milk) was introduced a loop of studied culture. Litmus milk acidified by S. thermophilus turns pink and then coagulates. After coagulation the colour remains pink due to very slow and often incomplete reduction of litmus, with a more intensely coloured upper ring.

Thermoresistance. Into 5 ml of skimmed milk by loop was introduced studied strain, incubated set at 37 °C for 4 h. After, the tubes were placed in the water bath at temperature of 60 °C for 30 minutes, and then cooled. Finally, the samples were incubated at 37 °C for 24 h. Acidified or coagulated milk means thermoresistance of studied strains.

Growth in the presence of sodium chloride. Inoculated tubes containing M17 broth with 20 $g \cdot l^{-1}$ and 40 $g \cdot l^{-1}$ NaCl incubated for 7 days at 37 °C. Presence of turbidity in the tube indicates the culture growth.

Growth in the presence of methylene blue. Inoculated skim milk supplemented with 0.1 $g \bullet l^{-1}$ and 1.0 $g \cdot l^{-1}$ of methylene blue solution were incubated at 37°C. Coagulation or its absence was visually determined at 12, 24 and 48 h. for 48 h. *S. thermophilus* does not grow in presence of methylene blue.

Growth at pH 9.6 was observed after 48 h of incubation at 37 °C in M17 broth which pH was adjusted to 9.6.

Isolation of EPS. 100 ml of yoghurt were centrifuged at 837 $rad \cdot s^{-1}$ for 10 min and 17 ml of trichloracetic acid (Chimprom, Ukraine) were added to each sample. Samples were cooled up to 4°C and again centrifuged at 837 $rad \cdot s^{-1}$ for 10 min. Precipitation of EPS from samples was provided using cold ethanol (1:3). The samples were kept in the fridge for 24 h and then centrifuged (40°C, 837 $rad \cdot s^{-1}$, for 10 min).

$(GTG)_5$-PCR fingerprinting

Total genomic DNA was extracted from the colony material of each strain after incubating them for 24 h in M17 broth (Merck, Darmstadt, Germany) at 37°C. DNA was extracted from 1 ml of the broth centrifuged at 5000×g for 10 min. A loop of culture was transferred to 1 ml of distilled water, mixed and centrifuged at 10000·g for 5 min. InstaGene suspension (Bio-Rad, Hercules, California, USA) was added to the sediment and incubated at 56 °C for 25 min. Then the mixture was vortexed, incubated at 100 °C for 8 min, vortexed, centrifuged at 10 000 ×g for 5 min and the supernatant containing DNA was removed. A volume of 2 µl of the DNA solution was added to the Polymerase Cain Reaction (PCR) reaction mixture (total volume, 25 µl) containing 10000 $nmol \cdot l^{-1}$ $(GTG)_5$ primer (5‘-GTG GTG GTG GTG GTG-3‘) synthesized by Qiagen (Hilden, Germany), 600 $\mu mol \cdot l^{-1}$ dNTP (Applied Biosystems, Foster City, California, USA), 1.5 $\mu mol \cdot l^{-1}$ $MgCl_2$, 1.5 U HotStarTaq DNA polymerase (Qiagen) and 2.5 µl of 10× concentrated PCR buffer supplied with the polymerase. PCR was carried out in a Biometra Personal thermal cycler (Whatman Biometra, Göttingen, Germany) using a thermal programmeconsisting of the initial denaturation at 95 °C for 15 min, 30 cycles of denaturation at 95 °C for 60 s, annealing at 40 °C for 90 s, ramping at 0.1 $°C \cdot s^{-1}$, and polymerization at 72 °C for 120 s, followed by the final polymerization at 72 °C for 10 min. A volume of 12 µl of the PCR

product was mixed with 1.5 µl of the loading buffer and analyzed by electrophoresis in a 1.5% agarose gel Seakem LE (FMC Bioproducts, Rockland, Maine, USA) for 5 h at 2.3 $V \cdot cm^{-1}$. Molecular size standard n×250 bp (Invitrogen, Carlsbad, California, USA) was electrophoresed in every fourth lane along with samples. The gel was stained with ethidium bromide for 30 min, destained in distilled water for 5 min, visualized under UV light and photographed with a digital camera. Rep-PCR profiles were compared with reference strains from culture collections.

Sample preparation for FTIR spectroscopy

S. thermophilus strains were cultured in pure cultures in M17 medium for 24 h at 37 °C. The cultures were decimally diluted with 0.9% NaCl and binary mixtures were prepared from the dilution 10^{-4}. A volume of 0.2 ml of the mixture was streaked on M17 agar and the plates were cultured for 72 h at 37 °C. Colonieswere picked directly from the plate by an inoculation loop, based on visual evaluation of colony morphology, and each was suspended in 100 µl of distilled water. The suspension containing whole cells was mixed by vortexing for 1 min and 35 µl of the suspension was pipetted on a position on a 96-position Zn-Se plate. The plate was dried for 45 min at 37 °C and immediately measured by Fourier transform infrared (FTIR) spectroscopy.

FTIR spectroscopy

A Bruker Tensor 27 FTIR spectrometer quipped with HTS-XT module (Bruker Billerica, Massachusetts, USA) was used. The spectrawere recorded in a range from 4 000 cm^{-1} to 400 cm^{-1}, with spectral resolution of 4 cm^{-1} and 32 scans per sample. The spectra were processed by OPUS software (Bruker Billerica, Massachusetts, USA), which involved averaging of 32 scans, calculation of first derivative by Savitzky-Golay algorithm with 9 smoothing points, and vector-normalization in the region from 1780 cm^{-1} to 720 cm^{-1}. Cluster analysis was carried out by OPUS software on the basis of Euclidean distances using Ward's algorithm, heterogeneities being assigned values from 0 to 2.

Strains employed

Six single-strain cultures were used for starter culture formation: EPS-producing Streptococcus thermophilus LB-50 and *Streptococcus thermophilus* LB-51, non-EPS *Streptococcus thermophilus* LB-52, *Streptococcus thermophilus* LB-53, *Streptococcus thermophilus* LB-54 and *Lactobacillus delbrueckii ssp. bulgaricus* LB-42 belonging to the National Collection of Non-Pathogenic Microorganisms of the Institute of Microbiology and Biotechnology (CNMN IMB), previously selected from raw milk. The selected strains were propagated three times consecutively using 0.1% inoculum volume in 10% reconstituted skim milk (InLac, Republic of Moldova) at 40°C until coagulation. Working strains (pure culture 10mL) were gradually associated by inserting into 30 mL of sterile skim milk and incubated at 40°C until coagulation (pH 4.6). Three mixed-strain starter cultures were used to make yoghurt samples for studying the influence of the EPS on the quality of the final product as follows: two EPS-starter culture consists of *S. thermophilus* CNMN-LB-50, CNMN-LB-51 şi *Lb. bulgaricus* CNMN-LB-42; *S. thermophilus* CNMN-LB-50, CNMN-LB-52 şi *Lb. bulgaricus* CNMN-LB-42 non-EPS starter culture – *S. thermophilus* CNMN-LB-52, CNMN-LB-53, CNMN-LB-54 şi *Lb. bulgaricus* CNMN-LB-42. Non-EPS starter culture served as control.

Yoghurt production

Samples of yoghurt were manufactured under industrial conditions using milk produced by JLC Group (Chisinau, Republic of Moldova). The milk was pasteurized at

71°C during 15 s and cooled down to 40°C. Two samples of yoghurt were produced: sample 1 using EPS-producing starter culture and sample 2 with non-EPS starter culture. Samples of yoghurt were incubated at 37°C until pH decreased to a value of 4.5, then kept at 4°C.

Milk acidification. To 10 ml of milk clot was added 20 ml of distillate water, 3 drops of phenolphthalein solution and agitated. Then titrated with a 0.1 $mol \cdot l^{-1}$ sodium hydroxide solution till the solution has been turned to pink. 1 ml of 0.1 $mol \cdot l^{-1}$ sodium hydroxide standard solution correspond to 0.009 g lactic acid.

Enumenarion of the total viable count of LAB was performend using standard pour plate technique. *S. thermophilus* and *L. delbrueckii subsp. bulgaricus* were incubated aerobically at 37 °C for 48 h. The total counts were expressed as log10 $CFU \cdot g^{-1}$ of the product.

Antimicrobial activity of selected strains was performed by using disc diffusion method. As the test-culture were used *Staphilococcus aureus* ATCC® 25932™, *Escherichia coli* ATCC® 25922™. Five sterile paper blank discs were placed on the agar plate which was inoculated by indicator strains and the filtered supernatant of *S. thermophilus* strains were applied on the surface of each discs. Plates were incubated at 37°C and zones of inhibition were observed. The inhibition zone was expressed in millimeters.

The viscosity of yoghurt samples was studied with a digital rheometer DV-III (BROOKFIELD, Canada) using the Rheocalc 32 software. Measurements were performed at different rotation speeds up to 21 $rad \cdot s^{-1}$. The viscosity measurement was carried out at a temperature of 25 °C.

Statistical analysis

Data were expressed as means ± standard deviations for triplicate determination. Statistical analysis was performed using Microsoft Excel 2007. Differences were considered to be significant at validity of α=0.95.

Results and discussion

The LAB selection for their use as starter cultures includes the strains isolation from natural sources and study of their properties, which determines the industrial value.

Milk is the most complete medium for selecting LAB. The use of sterile skimmed milk as a culture medium is more favorable for the growing of S. thermophilus. Thus, natural selection of industrial valuable strains occurs.

The *Streptococcus thermophilus* strains were isolated from cow's raw milk and spontaneous fermentation dairy product samples collected from local markets in different regions of the Republic of Moldova. Approximately 300 samples were studied for isolation of S. thermophilus

S. thermophilus, according to Bergey's Manual [7], are capable of developing at temperature between 42–45 °C. Though, the optimal growth is between 37–40 °C. Therefore all samples were cultivated at 37 °C. At the final of the routine examination of colony morphology and microscopy were selected 5 typical strains S. thermophilus for further research.

The 5 selected isolates were initially examined using morphological and biochemical tests (Table 1) and were classified as cocci growing in chains.

All isolates were able to grow at 20 $g \cdot l^{-1}$ of NaCl content and at 0.1 $g \cdot l^{-1}$ of methylene-blue in M17 broth. In litmus milk acidified by isolates were observed coagulation and reduction of pink colour. In additional 5 isolates fermented the lactose, saccharose, glucose and not fermented esculine. It is well known that S. thermophilus possesses a high ability to grow in a medium supplemented by lactose, glucose and saccharose [8, 9]. Inability to ferment esculine, high sensibility to 40 $g \cdot l^{-1}$ of NaCl content and lack of grow in 1 $g \cdot l^{-1}$ of methylene-blue distinguishes *S.thermophilus* species from *Enterococcus* genera [10]. According to the obtained results of morphological and biochemical tests the 5 isolates were identified as *S. thermophilus*.

Table 2

Morphological and biochemical characterization of selected isolates

Characteristics	Code of isolates				
	CNMN-LB-50	**CNMN-LB-51**	**CNMN-LB-52**	**CNMN-LB-53**	**CNMN-LB-54**
Cell morphology	Cocci in long chains				
Gram staining	+	+	+	+	+
Production of gas (CO_2)	-	-	-	-	-
Catalase production	-	-	-	-	-
Action in litmus milk	ACR	ACR	ACR	ACR	ACR
Thermoresistance	+	+	+	+	+
Growth in presence of					
NaCl 20 [$g \cdot l^{-1}$]	+	+	+	+	+
NaCl 40 [$g \cdot l^{-1}$]	-	-	-	-	-
methylene blue 0.1 [$g \cdot l^{-1}$]	+	+	+	+	+
methylene blue 1.0 [$g \cdot l^{-1}$]	-	-	-	-	-
pH 9.2	-	-	-	-	-
Glucose	+	+	+	+	+
Lactose	+	+	+	+	+
Sucrose	+	+	+	+	+
Galactose	-	-	-	-	-
Maltose	-	-	-	-	-
Ramnose	-	-	-	-	-
Manose	-	-	-	-	-
Sorbita	-	-	-	-	-
Esculin	-	-	-	-	-
Xilose	-	-	-	-	-

+= positive, -= negative
ACR – acidification, coagulation, reduction, Thermoresistance – growth after heating for 30 min at temperature 60 °C.

The PCR product visualization of $(GTG)_5$ PCR fingerprints are presented in Figure 1.

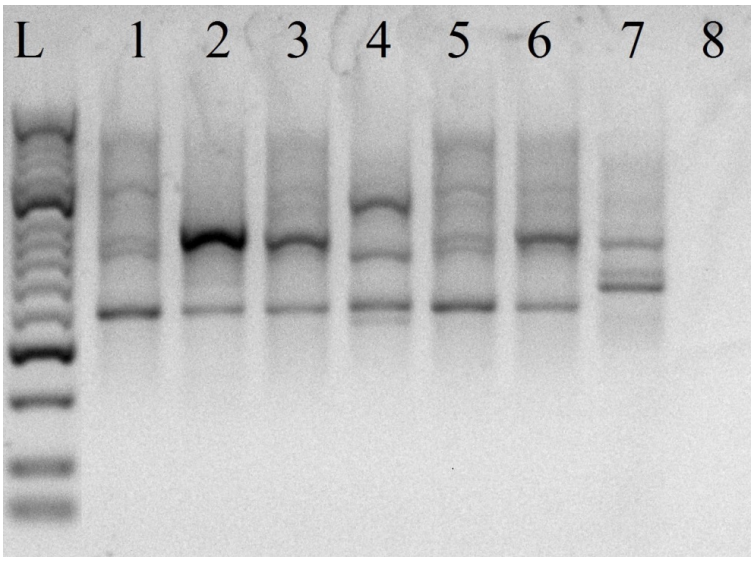

Figure 1. PCR products for *S.thermophilus* isolates
L – HipperLadder 50 bp; 1 – *S. thermophilus* CNMN-LB-52; 2 – *S. thermophilus* CNMN-LB-50; 3 – *S. thermophilus* CNMN-LB-51; 4 – reference strain *S. thermophilus*A737; 5 – *S. thermophilus* CNMN-LB-54; 6 – *S. thermophilus* CNMN-LB-53; 7 – positive control (*S. thermophilus*1241); 8 – negative control

DNA fragments were yielded 2 to 7 bands. The results confirmed that the similarity level of the genes of the studied strains was 99% with the reference strains of *S. thermophilus*.

The FTIR spectra of the 5 isolates were compared with the reference strain *S. thermophilus* A737. The Pearson product-moment correlation coefficient was applied to the studied strains across the spectra region from 4000 cm^{-1} to 500 cm^{-1} [11]. Naumann established that the highest strain correlation is reached at the following intervals: from 3000 cm^{-1} to 2800 cm^{-1}, 1500 cm^{-1} to 1400 cm^{-1} and 900 cm^{-1} to 700 cm^{-1} [12]. According to this study, the correlation of 5 isolates showed 99% similarity with reference strain.

After isolation and identification all 5 isolates of *S. thermophilus* were deposited at National Collection of Non-pathogenic Microorganisms of Institute of Microbiology and Biotechnology (CNMN IMB).

The next step of our study was determination of technological properties of the selected strains and their suitability for use as starter cultures. The fermentative activity of lactic bacteria is the most important technological criteria for the appreciation of their use in dairy industry. The intensification of the milk fermentation process and improvement of dairy products quality can be achieved by using biochemically active LAB strains.

The evolution of acidification was monitored for all selected S. thermophilus strains, as shown in Figure 2.

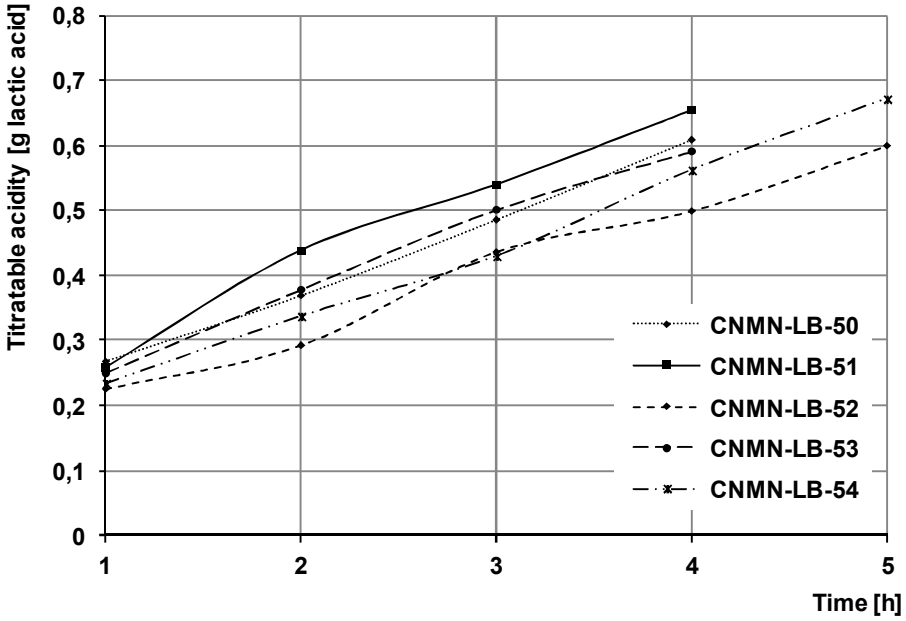

Figure 2. Kinetics of titratable acidity of the *S. thermophilus* strains in milk

Data shows that the selected strains of *S.thermophilus* has a high speed of the milk acidification for 5 hours. The selected strains had a different capacity of lactose fermentation. *S. thermophilus* CNMN-LB-50, CNMN-LB-51, CNMN-LB-53 coagulate skim milk in a 4±0.5 h, *S. thermophilus* CNMN-LB-52 and CNMN-LB-54 in 5±0.5 h.

The strain of *S. thermophilus* along with *Lb. bulgaricus* are used as starter culture for yoghurt production. The main pathogens in the dairy industry are *Escherichia coli*, *Staphylococcus aureus*, *Clostridium botulinum*, Listeria *monocytogenes*. These spoilage bacteria are capable to survive during a production process. *E. coli* and *S. aureus* in dairy products result from post-pasteurization contamination [13]. Therefore, one of the basic parameters for technological interest of LAB selection is their antimicrobial activity. The antimicrobial activity against *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 of 5 selected *S. thermophilus* strains were investigated using the agar-discdiffusion method [14] (table 2).

Table 2

Antagonism of *E. coli* and *S. aureus* by *S. thermophilus* isolates using agar-disc diffusion method

Code of strain	*Escherichia coli*	*Staphylococcus aureus*
	Zone of inhibition [mm]	
CNMN LB-50	16±0.5	20±0.5
CNMN LB-51	18±0.3	20±0.3
CNMN LB-52	17±0.2	19±0.3
CNMN LB-53	16±0.2	19±0.2
CNMN LB-54	17±0.5	21±0.5

Based on the results, a total of 5 analyzed strains of S. thermophilus showed inhibitor properties against conditional pathogenic bacteria *E. coli* and *S. aureus*. Sterile area varies between 16–18 mm against *E. coli* and 19–21 mm against the *S. aureus*. This indicate the functionally properties of selected strains.

Many thermophilic LAB are capable to produce exopolysaccharide (EPS) that play an essential role in texture forming of fermented products [15]. Therefore EPS producing capacity of selected *S. thermophilus* strains are important technological property. In this study were investigated all five selected strains on ability to EPS production.

As a result of the researches, two strains producing EPS were shown: *S. thermophilus* CNMN-LB-50 520±10 $mg \cdot l^{-1}$ and *S. thermophilus* CNMN-LB-51 436±10 $mg \cdot l^{-1}$. In the case of strain *S. thermophilus* CNMN-LB-52, the amount of EPS was low – 24±5 $mg \cdot l^{-1}$. The other 2 strains produce EPS or their amount are very small, and probably co-precipitate with the proteins upon of trichloroacetic acid addition and can no longer be detected after precipitating with ethanol. In general, the EPS yield of strains ranges from 2 $mg \cdot l^{-1}$ to 600 $mg \cdot l^{-1}$ in milk environment [16–18] and the EPS synthesis capacity is individual for each strain of the same taxonomic group.

On the basis of the obtained results were developed 2 starter cultures for yoghurt production: EPS-producing, non-EPS-producing starter culture, as a control sample served the yoghurt fermented by industrial starter culture (*S. thermophilus* and *L. bulgaricus*). 3 sets of yoghurt were manufactured in the industrial conditions. The results obtained for the yoghurt samples produced using starter cultures with selected *S. thermophilus* strains and industrial curter culture (table 3) showed that the developed starter culture are suitable to be used in yogurt production.

Table 3

Characteristics of yogurt samples

Parameters	**Yoghurt EPS**	**Yoghurt non-EPS**	**Yoghurt industrial starter**
Fermentation time [h]	4.0±0.1	4.2±0.1	5.0±0.1
LAB cout [log10 $CFU \cdot g^{-1}$]	8.8±0.2	8.4±0.3	8.3±0.2
Titratable acidity [g lactic acid]	0.645±0.01	0.633±0.01	0.626±0.01
EPS [$mg \cdot l^{-1}$]	651±10	-	-

Furthermore, developed starter culture possessed the highest fermentation activity comparative with the control sample. The viable count of LAB was at the same level for all samples. No difference was observed between titratable acidity between samples. In additional, the use of EPS starter culture increase the apparent viscosity that improve the consistency of yoghurt due to microstructure formation where EPS binds free water with the lipid and protein complexes of the product [19, 20].

Conclusion

As a result of the presented research was demonstrated the possibility of isolation from Moldavian raw milk and dairy products of spontaneous fermentation of autochthonous S. thermophilus strains with valuable biotechnological properties for industrial application, such as high antibacterial activity and EPS-producing capacity. The five selected strains of *S. thermophilus* showed characteristics adequate for their use as starter culture for yoghurt production. Developed starter cultures characterized by intense acidification of milk 4.0±0.2 h comparative with the industrial starter. The inclusion of EPS S. thermophilus strain clearly improved the apparent viscosity of yoghurt.

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