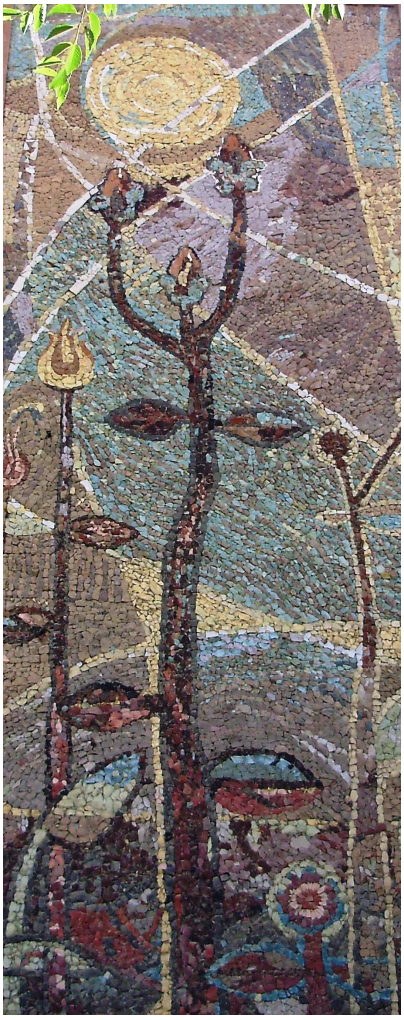

Young Scientists' Conference

New Aspects in Molecular Biotechnology and Biochemistry

27-28 June, 2013

H. Buniatian Institute of Biochemistry
NAS RA, Yerevan, Republic of Armenia
http://aab.sci.am

Dear colleagues,

H. Buniatian Institute of Biochemistry NAS RA and the Armenian Association of Biochemistry are pleased to announce the International Young Scientists Conference “The New Aspects in Molecular Biotechnology and Biochemistry”.

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Duration: 2 days

Place: Yerevan, Armenia

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The main scientific topics of the conference include biochemical, molecular and cellular biology as well as related and cross-linked fields of biomedical sciences.

Working language: English, Russian, Armenian

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Oral / Poster presentations. Duration of the oral presentation should be 10-15 minutes.
Working languages of the conference are English, Armenian and Russian.
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The materials of the Conference will be published in the Electronic Journal of Natural Scienses. Please send your article(s), prepared according to the "Instructions for manuscripts submission" (see below), to the e-mail address conference2013@list.ru.

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Young Scientists' Conference

NEW ASPECTS IN MOLECULAR BIOTECHNOLOGY AND BIOCHEMISTRY

27-28 June, 2013

H. Buniatian Institute of Biochemistry NAS RA
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Young Scientists' Conference

NEW ASPECTS IN MOLECULAR BIOTECHNOLOGY AND BIOCHEMISTRY

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Abstract must be in English, Russian, Armenian and no longer than a half of A4 page (*200-300 words*).
The article must not exceed 2-6 A4 pages, including figures (*not necessarily*) and References. The article may be written in English.

Please use “Times New Roman” font, with font size 12 (except References, where the font size must be 10). Line spacing should be 1.5. Please do not use text formatting and hyphenation. Latin terms must be in *Italic*. The number of references should not exceed 10. References should be cited in the text by sequential numbers in square brackets, e.g. `[1]', `[2, 7]', etc. At the end of the paper references should be listed in alphabetical order, in the style shown in the examples below, preceded by the number.

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Abstract (no more than half page, in English only; font size 12);
Keywords (up to 5 keywords, in English; Italic, font size 12);
Text of the manuscript, including:
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1(12), 2009

Microbiology *Մանրէաբանություն* *Микробиология*

This article has been reported at The International Conference "Enteric Bacteria and Inflammatory Bowel Disease (EBIBD)"

METHODS OF MOLECULAR IDENTIFICATION AS IMPORTANT TOOLS FOR CONTROL AND CERTIFICATION IN MICROBIOLOGY OF LACTIC ACID BACTERIA

O. V. Piksasova [*], M. A. Kornienko [**], Yu. D. Tsygankov [**], A. I. Netrusov *

**Biological Faculty, Lomonosov Moscow State University (MSU), Department of Microbiology, Biological Faculty, Leninskie gory 1, build. 12, Moscow, 119992 Russia;*
Phone: 007(495)-939-27-63; Fax: 007(495)-939-27-63; e-mail: piksasovaolga@gmail.com;
***State Research Institute of Genetics and Selection of Industrial Microorganisms, Pervyi Dorozhnyi pr. 1, Moscow, 117545 Russia*

ABSTRACT

Lactic acid bacteria, e.g. *Bifidobacterium* spp., *Lactobacillus* spp., *Streptococcus thermoplilus* etc., are commonly used for producing dairy and meat fermented products, in medicine as probiotics etc. and requires staying under tight control along each step of strain utilization. Lactic acid bacteria have a long history as industrial starters and therefore generally recognized as safe (GRAS). Although streptococci are not GRAS organisms, *Streptococcus thermophilus* are considered as food-grade and originally used for yogurt, sour-cream and other dairy production must be obeyed the same laws as other GRAS microorganisms. The methods for strain tracing must be precise and reliable. As phenotyping and serotyping rely on gene expression and, consequently, may be unstable and result in ambiguous speciation, DNA-based characterization is now widely accepted. Several molecular typing methods are presently available and some of them have been applied to oral streptococci This study was done to determine whether fingerprinting techniques, random amplified polymorphic DNA (RAPD) analysis and pulsed-field gel electrophoresis (PFGE) are useful to identify and even classify oral streptococci on strain level. DNA was prepared and purified from 25 strains of thermophilic streptococci including 2 reference strains of *Streptococcus thermophilus* and from 23 fresh isolates of *S. thermophilus* from various fermented dairy products. DNA amplification was primed with each of three arbitrarily selected primers 15 to 22 nucleotides in length. The amplified DNA fragments (amplicons) obtained were compared by agarose gel electrophoresis. For PFGE analysis was used *SmaI* restriction endonuclease. DNA patterns were obtained on a contour hexagonal pulsed electric field in agarose gel. Pending the analysis of numerous other strains, the data suggest that RAPD may be of value: (i) to distinguish the species *S. thermophilus* potentially from other species of LAB, (ii) to differentiate and possibly classify thermophilic streptococci on intraspecies level and (iii) as a valuable tool in lactic acid bacteria controlling, by virtue of its rapidity, efficiency and reproducibility in generating genetic fingerprints of streptococcal isolates.

Key words: *LAB, RAPD-PCR, PFGE, intraspecific divergence*

INTRODUCTION

Lactic acid bacteria are traditionally used as starter microorganisms for dairy products with health promoting effect. While probiotics seem not to have a major effect in altering the composition of the gut microbiota, they may have a role in manipulating the immune system in relation to specific diseases that have an immunological etiology, such as inflammatory bowel diseases and allergies [6]. It was reported that ingested lactic acid bacteria partially survive in stressful conditions of gastric acidity and bile salts and therefore pass live through the gastrointestinal tract [2] where they may influence the content of endogenous microflora. *Streptococcus thermophilus* as soon as *Lactobacillus delbrueckii* subsp. *bulgaricus* have been classically used as starters for milk fermentation in yogurt production. The concentration of these organisms in the human or animal gastrointestinal tract has been poorly examined in comparison with that of other probiotic strains.

In purpose of strain tracing during all steps of its utilization accurate techniques should be determined and used. Traditional phenotyping and serotyping may give unstable results because of relying on gene expression therefore it took place the development of typing methods based on the microbial genotype or DNA sequence, which helps minimizing problems with typeability and reproducibility and, in some cases, enable the establishment of large

databases of characterized organisms [4]. Molecular typing methods allow differentiation of microorganisms on genus, species and strain levels. Recently it was developed a number of new DNA typing methods which add and somewhere replace traditional typing with microbiological tests. There are such methods in this list as DNA–DNA hybridization, restriction endonuclease analysis (REA), multilocus enzyme electrophoresis (MLEE), ribotyping, PCR and colony hybridisation with DNA probes. A PCR-based procedure of DNA fingerprinting, called arbitrarily primed PCR (AP-PCR) or randomly amplified polymorphic DNA (RAPD) analysis, has been applied to strain identification and typing of several groups of bacteria including streptococci [7]. The present study used RAPD analysis and PFGE for typing *S. thermophilus* strains and to explore the potential of this typing method to distinguish these from the other taxa that comprise the group of thermophilic streptococci as soon as within species *S. thermophilus* itself.

Streptococcus thermophilus is an available microorganism which can be used as model LAB bacteria for both applications, biomedical and fundamental.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All 24 strains of *Streptococcus thermophilus* were isolated from fermented dairy products (yoghurt, sour cream, cheese, sour clotted milk, cottage cheese, and sour milk), manufactured in different regions of Russia, as well as in Italy, Croatia, Slovenia, and France. All strains used in the present study are listed in *Table 1*. Two cultures, B7637 and ATCC19258, provided by the All-Russian Collection of Industrial Microorganisms were used as reference strains of *S. thermophilus*. Strains were grown aerobically in MRS broth (Oxoid) and M21 broth [1]. All cultures were incubated at 42°*C*.

Table 1. The list of used bacterial strains with notification of source of origin and region.

Isolate №	Source product	Region
BC 201	Sour-milk drink	Croatia
BC 216	Fermened milk beverage	Croatia
BC 221	Sour-milk product	France
BC 223	Sour-milk product	France
BC 231	Sour-milk product	France
BC 233	Sour-milk product	France
BC 236	Sour-cream	Russia
BC 247	Sour-milk product	Slovenia
BC 250	Fermened milk beverage	Croatia
BC 257	Starter	Belorechensky dairy factory, Irkutsk region
BC 259	Kurunga (national sour-milk product)	Buryatia
BC 310	Sour-milk product	Croatia
BC 311	Sour-milk product	Croatia
BC 313	Sour-milk product	Croatia
BC 314	Sour-milk product	Croatia
BC 316	Sour-milk product	Croatia
BC 317	Sour-milk product	Croatia
BC 322	Sour-milk product	Italy
BC 323	Sour-milk product	Italy
BC 324	Sour-milk product	Italy
BC 332	Sour-milk product	Astrackhan
BC 337	Sour-milk product	Italy
BC 338	Sour-milk product	Italy

Phenotypic characterization. All strains were characterized by morphology and simple physiological tests. Ability to grow at different temperatures was determined in M21 broth at 10°*C* for a week and at 45°*C* for 48 h. Growth in the presence of 2 to 6% of NaCl was observed in M21 with salt addition. The ability to grow at different pH and to produce lactic acid was tested in sterile skimmed milk at 42°*C* for 168 h. pH was measured with electronic pH-meter "Mettler Toledo MP220". Ability to esculine fermentation and exopolisacharide production was also estimated for all strains (data of phenotypic characterization was taken into account but not shown here).

Molecular techniques. Genomic DNA was extracted using the "DNA-sorb-AM" purification kit (AmpliSens, Moscow, Russia) according to recommendations of manufacturer or single colonies of the different strains were employed as templates.

PCR reactions were performed at a Tercik MC2 DNA thermal cycler (DNA technology, Moscow, Russia) with AmpliSens- 200-1 kit with *Taq* polymerase (AmpliSens, Moscow, Russia). PCR mixtures were prepared as following: 2-5 μL of genomic DNA, 300 μM of dNTPs, 6 μL of 5X PCR buffer containing 0.06 μL of 5% solution of Na aside, 0.9 μM of each *primer* and 1 U *Taq* of DNA polymerase (AmpliSens, Moscow); distilled water was added to complete the final volume of the reaction.

For PCR the following primers were used, **8f**: 5'- agagtttgatcctggctcag – 3' [Boyer et al., 2001], **1492r**: 5'- ggttaccttgttacgactt – 3' [3]. The primers were synthesized using the Biosset ASM-800 DNA synthesizer. These primers were designed for amplification the whole sequence of 16S rRNA gene region of lactic acid bacteria. The length of expected amplification product is 1484 bp.

RAPD-PCR assay. The RAPD-PCR conditions used with primers M13, 5'-gagggtggcggttct-3' [4]; MSP, 5'-gtaaaacgacggccagt-3' [9]; and ERIC-1, 5'-atgtaagccttcggggattcac-3' [10] were carried out was carried out using reagents from PCR-DNA kit (AmpliSens, Moscow) and primers synthesized using the Biosset ASM-800 DNA synthesizer. Amplification temperature profile was as following: 94°*C* for 120 s, 40 cycles of [94°*C* for 30 s; 45°*C* for 30 s; 72°*C* for 80 s], 72°*C* for 4 min (the duration of the whole program was 2 h 26 min).

PFGE assay. Preparation of samples for PFGE was as following: bacterial biomass suspended in physiological salt solution and centrifuged at 10000 *g* for 3-5 minutes. The pellet was resuspended in 30 *mkl* of distilled water and mixed with 180 *mkl* of hot 2% agarose (Sigma). This melted mix was used for preparation of agarose bricks. The bricks were incubated for 20 minutes at room temperature and afterwards for 20 minutes at 4-8°*C*. The cell lysis is being produced directly in slices of DNA containing agarose plugs during incubation for 8-9 hours at 37°*C* in the solution containing lysozyme in concentration of 1 mg per ml and 10mM of EDTA. Next step included treatment of DNA with proteinase K at 53°*C* for 16 hours. After that DNA blocks should be properly washed with distilled water and can be saved for some time in sterile conditions at 4°*C* in 0.5*M* EDTA. Restriction was produced following the traditional protocols [Maniatis, 1984] in the presence of *Sma*I restriction endonuclease and restriction buffers (Fermentas) according to recommendations of the manufacturer.
DNA fragments were separated by PFGE in a 1% agarose gel (type A, Sigma) that was prepared and run in an 0.5× Tris-borate-EDTA buffer on a contour hexagonal electric field (CHEF-DRIII, BioRad). The pulse of +6V/cm with gradual alteration of pulse duration from 5 to 15 seconds during 24 hours was applied. The buffer during the electrophoresis was being recirculated and the temperature of buffer was maintained at 14°*C*. The electrophoresed DNA was stained in the gel with ethidium bromide (1 *ttg/ml*) and was visualized using an ultraviolet transilluminator equipped with a standard gel photography apparatus.

Use of cluster analysis for the classification of RAPD-PCR and restriction patterns. Registration of the RAPD electrophoretic patterns, normalization of the densitometric traces, grouping of strains by the Pearson product-moment correlation coefficient (*r*) and UPGMA cluster analysis were performed by techniques described by Pot et al. [5] using the software package BioNumerics (Applied Maths, Kortrijk, Belgium). Similarities between strains were estimated with Dice coefficient. Clustering of the pairwise distance matrix among molecular profiles was performed using the unweighted pair group method with average linkage (UPGMA).

16S rRNA gene sequence analysis. Each PCR was performed with primers 8f and 1492r (see above) to amplify 16S rDNA (positions 8-1492 in the *Escherichia coli* numbering system). The PCR products were purified using kit GenElute Minus EtBr Spin Columns (Sigma) according to manufacturer recommendations. The purified DNA was used for 16S rDNA sequence analysis performed with an automatic DNA analyzer CEQ8000 Beckman-Coulter (Beckman) and primers 8f as the sequencing primer. The assembled partial rDNA sequences were compared with sequences in the GenBank database [4]. The GeneBank sequence AY188354 (*S. thermophilus* ATCC19258) was used as reference. Comparative analysis was performed with data from NCBI database (http://www.ncbi.nlm.nih.gov/blast). Sequencing results were collected and clustered using package software Mega2 in Internet (http://www.megasoftware.net) and BioNumerics (Applied Maths, Kortrijk, Belgium).

RESULTS AND DISCUSSION

In current study it was made an attempt to create a multiparametric library of lactic acid bacteria on isolates of *Streptococcus thermophilus*. The 24 isolates from natural sources (self-fermented dairy products, plants' surface and mammals) were used. We investigated the interest of the composition of RAPD-PCR protocols with different primers and PFGE for certification of streptococci strains. All isolates were amplified and analyzed with *Sma*I endonuclease. All results of molecular testing as well as data of biochemical and phenotypic analysis were collected for every single strain to provide certification of bacteria.
The method of pulsed-field electrophoresis (PFGE), randomly amplified polymorphic DNA analysis (RAPD-PCR) and gene sequencing were used for characterization of isolates. Primer M13 was designed for minisatellite sequence of bacteriophage M13. As it was shown earlier, most bacteria contain plural repeats of satellite sequence of M13 [8, 9]. Primer ERIC-1 was designed for the 126 bp region located outside the coding region of bacterial genome, and contained highly conservative central inverted repeat [4]. Primer MSP earlier was used for identification and differentiation of *Bifidobacterium* sp. The convergence of analyzed strains with typical strain of *S. thermophilus* estimated with RAPD-PCR method is presented in quantitative equivalents (%). Results are shown in surface dendrograms and three-dimensional charts (*Fig. 1a, 1b*). Cluster analysis of RAPD-PCR electrophoretic patterns was carried out by the Pearson correlation with the Corr procedure showed high level of interspecies divergence of *S. thermophilus*.
It was demonstrated that two-dimensional dendrograms most adequately reflected the relatedness and interrelations of the system components (strains within clusters). At the same time, three-dimensional dendrograms provided

estimation of a distance between the total data body and the deviated component. Numerical analysis of the patterns obtained with primer M13, provided identification of seven clusters. The first, most abundant cluster, included the *S. thermophilus* reference strain ATCC19258, along with BC216, BC250, BC231, BC223, BC310, BC233, BC221, BC201, BC247, BC236, BC259, and BC257. The second cluster was formed by the strains BC323, BC322, BC337, BC332, and BC329. The third cluster consisted of the strains BC324, BC338, and BC316, and the fourth cluster contained the strains BC311 and BC317. The fifth, sixth, and seventh clusters were composed of single strains of BC313, BC314, and B7637, respectively. The level of similarity within one cluster ranged from 77.3 to 99.4%, and between the strains from different clusters, from 37 to 63.5%. RAPD-PCR with primer M13 showed the lowest reproducibility at the range of 70% between the three variants (*Fig. 2a, 2b*). The high level of polymorphism among the patterns obtained with primer M13 provided differentiation of the bacterial isolates at the strain level.

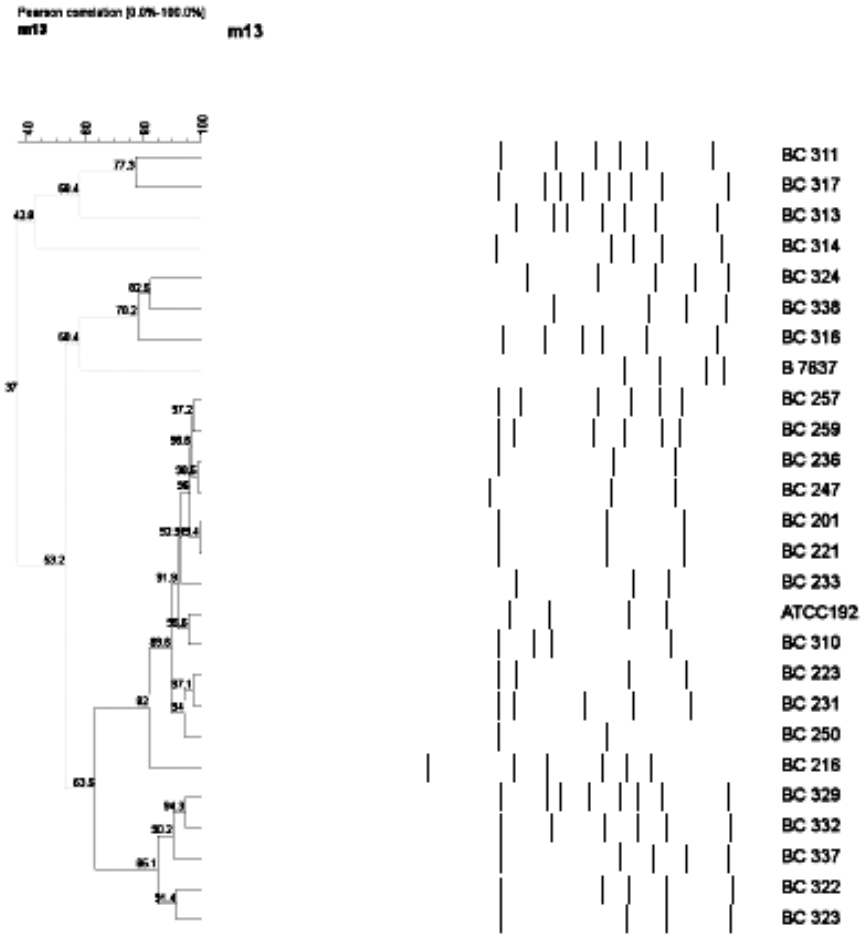

Fig. 1a. The clusters of RAPD patterns of 25 strains of thermophilic streptococci obtained with primer M13. Strain designations are shown to the right from the lanes. The dendrogram branches with similarity level under reproducibility threshold (lower 70% here) are shown as single clusters and marked with dash line. The dendrogram branches with similarity level over reproducibility threshold are drawn with firm line. For each branch the magnitude of cofenetic correlation is demonstrated. The 1-kb scale of DNA fragment molecular size is shown above the clusters.

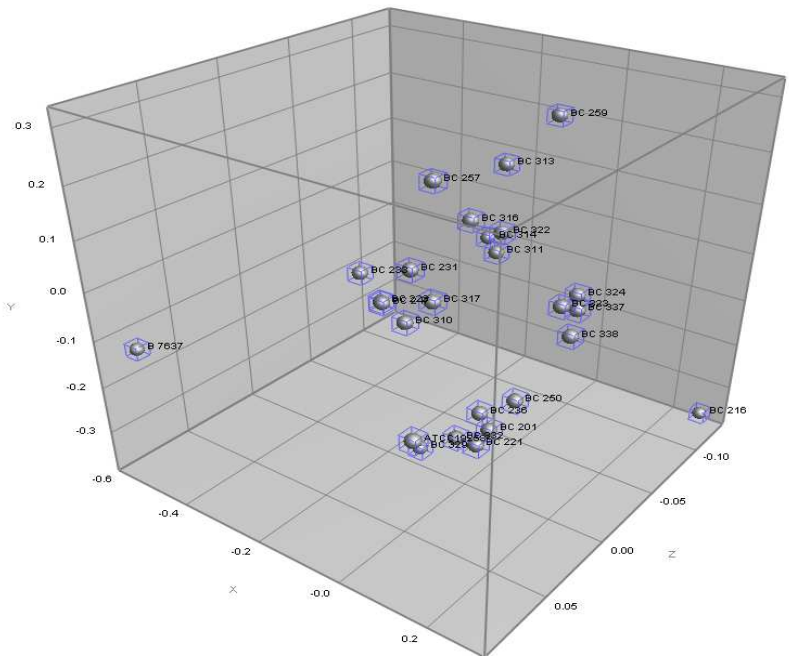

Fig. 1b. The clusters of RAPD patterns of 25 strains of thermophilic streptococci obtained with primer M13 displayed as three-dimensional chart and designed with use of Pearson coefficient.

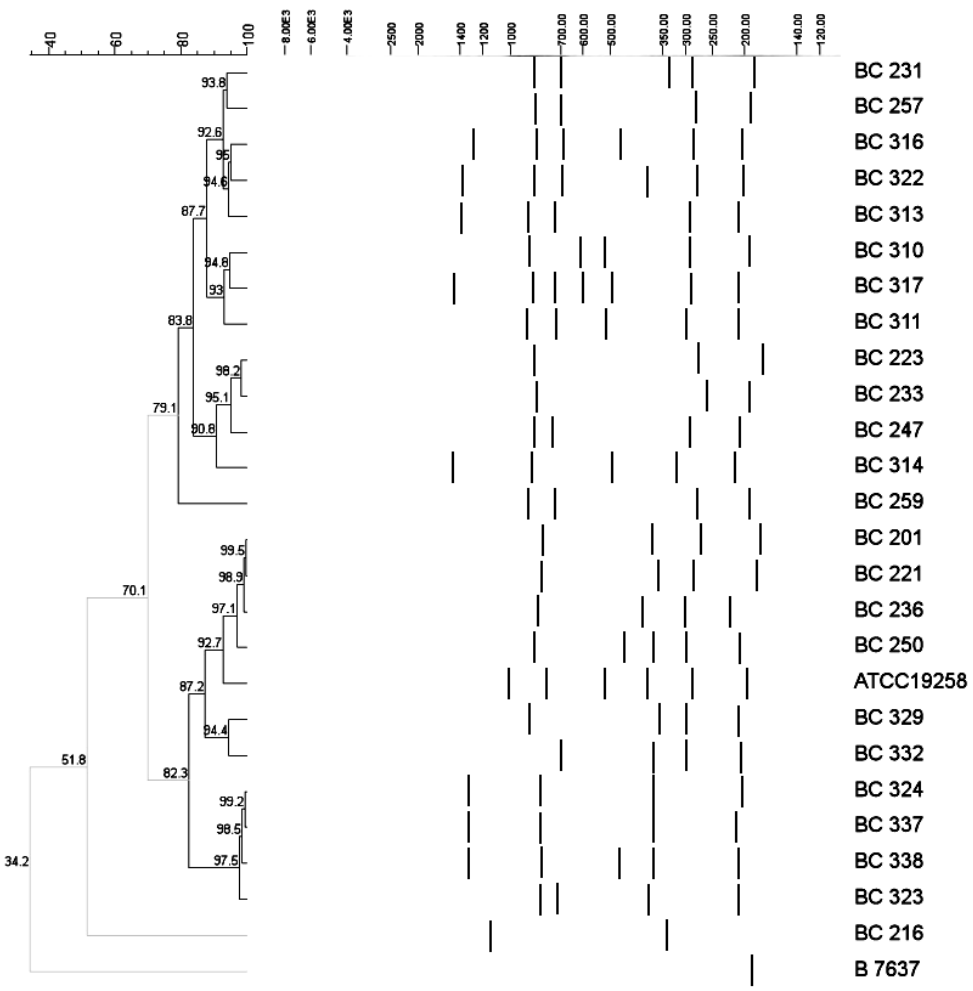

Fig. 2a. The clusters of RAPD patterns of 25 strains of thermophilic streptococci obtained with primer MSP.

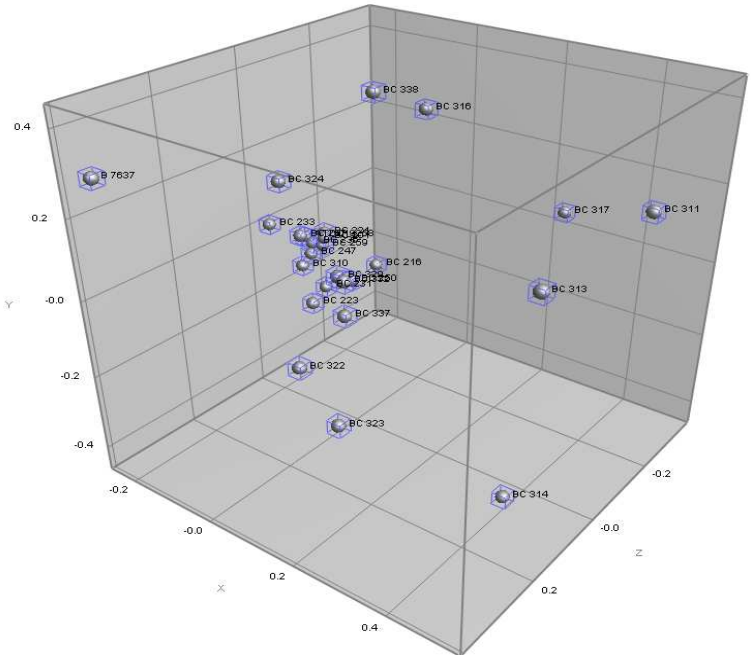

Fig. 2b. The clusters of RAPD patterns of 25 strains of thermophilic streptococci obtained with primer MSP displayed as three-dimensional chart and designed with use of Pearson coefficient.

Numerical analysis of patterns obtained with primer MSP. Four main clusters were found at the 80% similarity level (Fig. 3a, 3b). The level of similarity within one cluster ranged from 79.1 to 99.5%. The first cluster included the reference *S. thermophilus* strain ATCC19258 along with the strains BC323, BC338, BC337, BC324, BC332, BC329, BC250, BC236, BC221, and BC201; the second (similarly abundant) cluster was formed by the strains BC259, BC314, BC247, BC233, BC223, BC311, BC317, BC310, BC313, BC322, BC316, BC257, and BC231. Almost all *S. thermophilus* strains were found in clusters 1 and 2 with the exception of strains B7637 and BC216 (clusters 3 and 4, respectively). Reproducibility of RAPD-PCR with primer MSP was 75%. MSP represents rather conservative region of bacterial genome. The fragment electrophoretic patterns are similar. Substantial deviation from the total strain set was demonstrated by the strains BC216 and B7637 with the similarity levels of 51.8 and 34.2%, respectively. The ability of first strain to galactose and ribose fermentation distinguishes it from the reference representatives of *S. thermophilus*. BC216 was also capable to exopolysaccharide production. Physiological and biochemical properties of the second strain were indistinguishable of those of the reference strains. Therefore, the use of RAPD-PCR with primer MSP and cluster analysis would allow the identification of *S. thermophilus.*

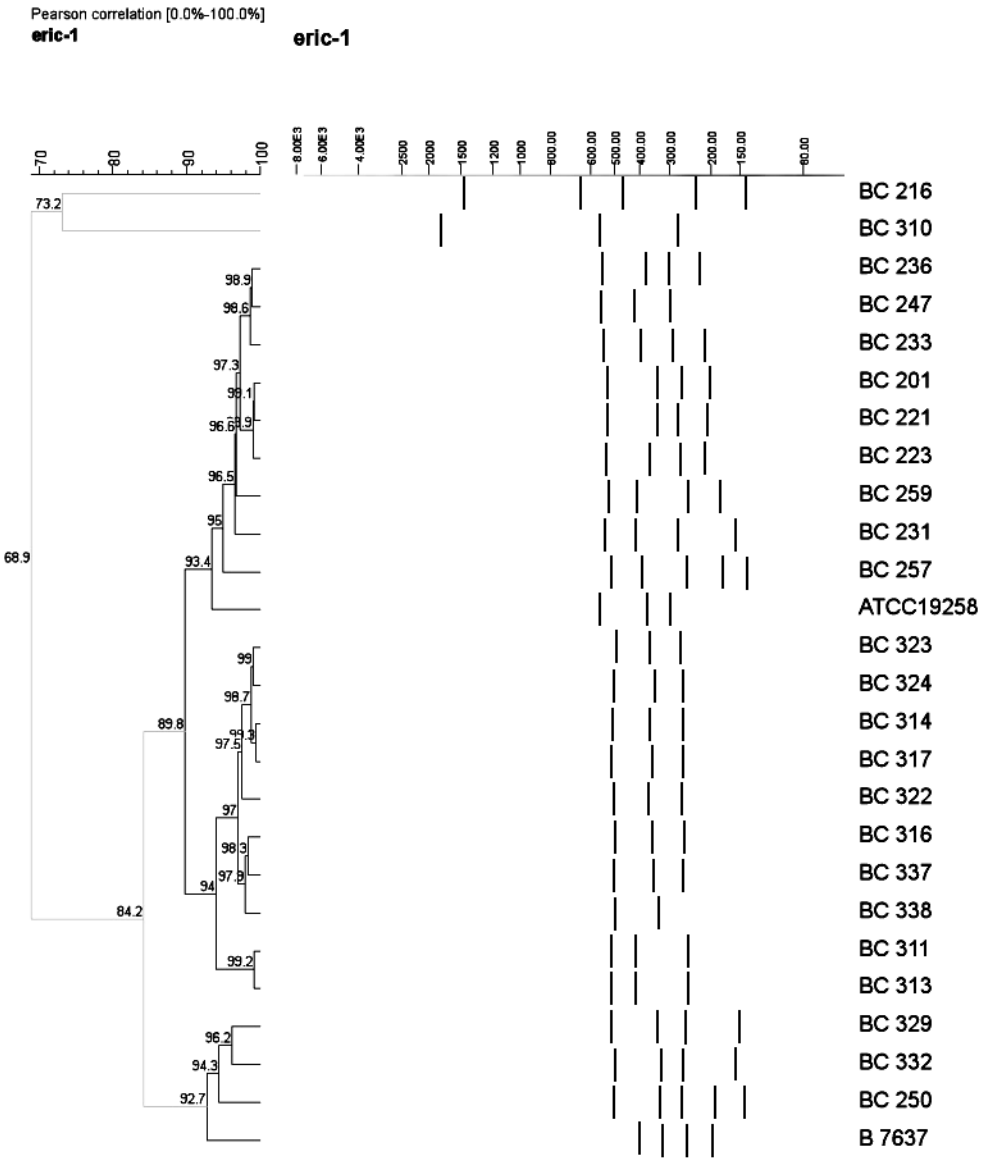

Fig. 3a. The clusters of RAPD patterns of 25 strains of thermophilic streptococci obtained with primer ERIC-1.

The RAPD-PCR variant with primer ERIC-1 characterized by highest reproducibility of 89% (*Figs. 3a, 3b*) showed two homogenous clusters at the similarity level of 90% and within the clusters ranging from 89.8 to 99.3%. The first cluster was formed by the strains B7637, BC250, BC332, and BC329. The second cluster included all the remaining strains, except BC310 and BC216, which formed the third and fourth clusters, respectively. The fingerprint patterns are more homogenous than in two previous variants of RAPD. The reason is higher conservative region of genome. All strain fingerprints (except for BC310 and BC216) contained the 600-bp fragment. Thus, two strains out of the whole collection examined, formed a separate cluster. The similarity level of 73.2% was between these strains, and 68.9% between these strains and the others. It is suggested that ERIC-1 genome region is the most suitable for identification of the bacteria at higher taxonomic levels, such as species level and higher. For this reasons, it is considered that RAPD-PCR with primer ERIC-1 can be used for the identification of bacterial isolates as an additional test (along with the analysis of the 16S RNA gene structure) for the belonging of the strains examined to *S. thermophilus.*

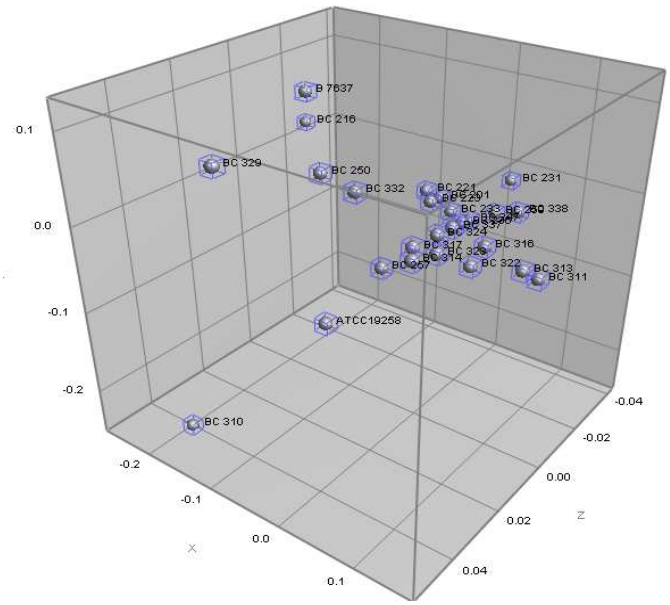

Fig. 3b. The clusters of RAPD patterns of 25 strains of thermophilic streptococci obtained with primer ERIC-1 displayed as three-dimensional chart and designed with use of Pearson coefficient.

Restriction fingerprints obtained with PFGE were also included. Cluster analysis of restriction patterns was carried out according to Dice correlation. Reproducibility of PFGE with *SmaI* endonuclease for *S. thermophilus* was estimated as 70%. The most number of analyzed strains were joined in two main clusters (*Fig. 4*). Strain pairs BC257-BC259, BC311-BC313, BC310-BC337, BC322-BC323, BC231-B7637 gave absolutely identical restriction pictures. Restrictograms of other investigated isolates contained DNA fragments of close molecular weight (*Fig. 4*). The similarity level within one cluster ranged from 71 to 86.5%. Such low similarity value between strains testifies that this method doesn't provide adequate results for relatedness of single strains to each other and for highly effective certification of strains of *S. thermophilus* of close origin. Molecular size of DNA fragments can be estimated using molecular ladders. Summarizing the weight of all fragments the genome size can be registered and will be an important feature of this certain strain. Genome size of investigated streptococcal strains varied from 1.417 Mb to 2.000 Mb (*Table 2*). Molecular weights (given in kb) and number of restriction fragment of genome DNA of investigated streptococci as soon as the total sums corresponding to the whole genome size are shown in *Fig. 4.*

Table 2. Restriction fragments sizes of genome DNA of *S. thermophilus* (conveyed in kb scale).

	BC 201	***BC 216***	***BC 221***	***BC 223***	***BC 231***	***BC 233***	***BC 236***	***BC 247***	***BC 250***	***BC 257***	***BC 259***	***BC 310***	***BC 311***	***BC 313***	***BC 314***	***BC 316***	***BC 317***	***BC 322***	***BC 323***	***BC 324***	***BC 329***	***BC 332***	***BC 337***	***BC 338***	***B 7637***	***ATCC 19258***
1	344	539	326	332	539	235	326	476	252	358	367	305	401	351	344	506	539	539	332	423	378	320	289	266	476	353
2	286	291	253	253	338	187	205	212	191	250	251	214	286	286	260	332	326	209	253	265	215	214	213	216	332	276
3	213	248	207	203	206	181	187	199	178	175	176	201	205	208	211	206	208	198	203	204	206	214	199	201	205	190
4	204	204	183	182	188	167	176	191	168	156	158	189	195	195	186	190	191	188	182	182	202	201	187	176	181	180

5	167	159	160	159	181	156	156	173	155	128	148	176	159	158	162	174	166	170	159	158	180	178	174	155	162	175
6	155	105	153	149	166	143	118	146	131	113	128	149	144	145	152	132	136	139	149	100	175	144	150	135	136	170
7	123	98	120	116	133	116	103	106	114	105	113	132	112	113	118	102	106	101	116	87	167	102	131	115	126	165
8	85	89	94	94	96	99	98	96	96	67	104	99	70	68	91	73	73	88	94	51	143	78	117	97	99	114
9	68	75	57	56	61	65	65	67	89	58	67	85	54	54	54	45	42	52	56	40	113	40	99	85	88	102
10	53	60	48	48	45	27	40	60	77	40	58	52	43	44	45		40	42	48		103	38	87	66	52	96
11	43	45	40	40	40	23	33	51	52			40	35	36	35		37	32	40		88	31	55	51	42	69
12		39				18	28	46	40			28									40		40	40	37	63
13							22	41	33												39					40
14							18														27					
Sum total (kb)	1741	1951	1641	1631	1993	1417	1576	1863	1576	1449	1570	1670	1704	1656	1658	1759	1863	1758	1631	1509	2075	1561	1741	1604	1936	1993

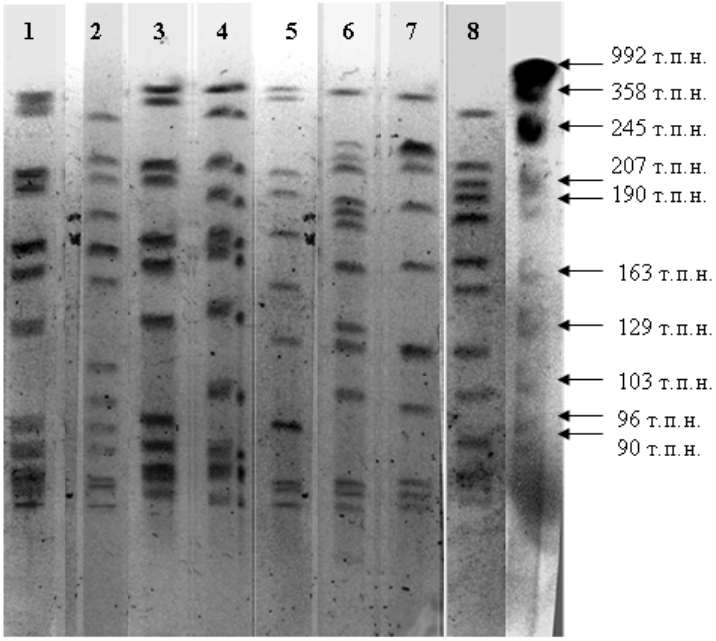

Fig. 4. Restriction fingerprints of genome DNA of strains from different similarity groups according to cluster analysis of restriction patterns obtained by PFGE with *SmaI* endonuclease: 1) BC311, 2) BC338, 3) BC313, 4) BC314, 6) BC329, 7) BC332, 8) BC310, the right lane – genome DNA restriction pattern of *E.coli* JC7623 obtained by PFGE with *NotI* endonuclease used as marker.

Strain affiliation was identified by partial sequencing (first 500 bp) of 16S rRNA gene. As control samples it were taken two typical strains of *S. thermophilus* ATCC19258 and B7637. Results of sequence comparison are shown in *Table 3***Error! Reference source not found.**. Homology level given in percentage was rated taking into account phylogenetic genetic distances which reflect variations of nucleotide mismatches per each 100 bp in determined 500 bp part of 16S rRNA gene of investigated strains. Homology range arranged from 99.5 to 100%. Maximum divergence at 99.4% has been shown for one strain, BC216.

CONCLUSIONS

As it was shown in this work the RAPD-PCR is an acceptable method for classification and typing, the patterns obtained from amplification with different primers may be used for identification on higher or lower phylogenetic level. Several primers can be combined. The procedures for reading the patterns, calculating similarity of distance measures, clustering the patterns require special equipment and software but can provide information for strain certification. RAPD-PCR with at least two separate primers coupled to hierarchical cluster analysis was a powerful and convenient tool for the classification and typing of thermophilic streptococci of dairy origin. This result may reflect a limitation to the use of a single primer. Use of RAPD-PCR for typing and investigation of intraspecific strain affiliation of *S. thermophilus* showed that similarity coefficients vary widely for RAPD-PCR variants with different primers. The widest diversity, from 77.3% to 99.4% within the range of one cluster and less than 70% between clusters of the diagram, can be seen on results of RAPD-PCR with primer M13. This variant can be used for intraspecific strain characterization. RAPD-PCR with MSP primer shared the group of investigated strains on more heterogeneous clusters with similarity coefficients range within one cluster from 79.1% to 99.5%. At the same time electrophoretic picture of patterns bored close similarity for 24 isolates that gives an evidence about lower sensitivity of this variant of RAPD to genome differences of strains in one species, that data confirms the corresponding genome region to be less subjected to genetic reorganizations. The third variant of the method,

RAPD-PCR with primer ERIC-1 also allowed separating two strains from the whole group of the strains. Cluster analysis gave two clusters with inner similarity coefficient from 89.8% to 99.3%. Stated data brought us to the conclusion that RAPD-PCR with primer ERIC-1 is the best match for certification of bacteria on higher phylogenetic levels than strain one and provides necessary data for rating the isolate to *S. thermophilus*. Moreover, when the purpose is identification rather than classification, cluster analysis is less appropriate for the interpretation of the results. The most precise method for identification of unknown strain remains sequencing of 16S rRNA gene. Even partial sequencing allows distinguishing of strains. Use of fingerprinting methods such as genome restriction and PFGE or RAPD-PCR should be used as reliable ones for strain certification and as an instrument for controlling strain reformations.

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