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IDENTIFICATION AND COMPARATIVE CHARACTERIZATION OF NEW LACTIC ACID BACTERIA ISOLATED FROM ARMENIAN DAIRY PRODUCTS BY PHENOTYPIC AND MOLECULAR METHODS

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More than 600 strain isolates were obtained from 200 matsun and salted cheeses samples. The strains were screened for potential probiotic properties, antibiotic and phenol resistance. Eight different lactic acid bacteria strains with high antibacterial activities were identified by multiphase approach comprising classical and molecular methods and indicated their relation to four species distributed in two genera Lactobacillus (*L. rhamnosus*, *L. helveticus*, *L. delbrueckii*) and Enterococcus (*E. faecium*).

Keywords: lactic acid bacteria, PCR identification.

Introduction. The great variety of ecological conditions and geographic landscapes of Armenia with its sharply defined vertical zoning, promoted the development of unique associations of lactic acid bacteria (LAB) in traditional dairy and fermented products prepared during millennia. The isolation of unique LAB communities from different fermented products, recognized for their significant physiological, antibacterial, antioxidant and antiallergenic activities, require the determination of the biological properties and the characterization of biotechnological potential of the strains presented among them because of the promising perspectives for the production of new biologically active substances. The traditional Armenian sour-milk product matsun is an example of product made by a unique association of LAB [1]. It is rich in proteins and possesses antibacterial and antioxidant activities. It is also applied in folk medicine since immemorial times and was used for curing postoperative wounds, skin burns etc. Many studies suggest that the regular use of matsun increases colonization resistance, intensifies immunity, suppresses allergic reactions, normalizes histamine and oxalic acid concentration, and exerts hypocholesterolemic, antitumor and other positive effects. The beneficial effects such as the decrease of pH, the competition for nutrients, as well the secretion of various antibacterial components, is at the base of inhibitory antibacterial action and can be attributed mainly to the presence of LAB, excreting substances with antibacterial activities such as hydrogen peroxide, carbon dioxide, diacetyl, organic acids (lactic acid in particular), fatty

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acids (antifungal components) and finally bacteriocins [2, 3]. The antibacterial activity of LAB can be also supplemented by the proteolytic activity due to the secretion of proteases and peptidases too.

The unambiguous identification of species using phenotypic methods such as sugar fermentation patterns, particularly within the genus Lactobacillus, may sometimes be very hard and complicated due to the increasing number of LAB species, which vary in small numbers of these features [4]. Nowadays Lactobacillus species can be much better identified using combinations of methods other than phenotypic ones. These include physical chemical methods or methods based on polymerase chain reactions (PCR) such as SDS-PAGE protein patterns [5], Fourier transformed infrared spectroscopy [6] or molecular genetic methods including DNA/DNA hybridizations [7], or 16S rRNA genes sequencing [8]. Thus, the main goal of the presented results is the possibilities of analysis with application of PCR based methods for rapid identification of LAB isolated from traditional dairy products, the deeper investigation of the lactic microbiota found in Armenian matsun, as well the selection of strains with promising beneficial potential for the dairy industry.

Materials and Methods.

Sampling and Isolation. More than 200 samples of matsun and cheeses have been collected from various mountain regions in Armenia during a period between 1999 and 2009. The type of the milk used for the preparation (cow, goat, sheep, buffalo or their mix) was also taken into consideration. The purification of these samples led to the isolation of more than 600 different strains of LAB. The latters were obtained by picking up single colonies after cultivation on selective nutrient media (MRS, M17, hydrolyzed milk, 10% skim milk "Himedia", India) in selective temperature conditions, and their purity was controlled by microscopic analysis of the obtained cultures. LAB were maintained in a viable condition by sub culturing them once a month in enriched milk (10% skim milk, 0.1% peptone, 0.1% yeast extract, pH 7.0) or in MRS medium, and saved at $-20^{\circ}C$ (MRS media, 20% glycerol).

Phenotyping Analysis. Cell morphology and motility were determined by light microscope Olympus CH2 (Japan). Gram staining, investigation of the optimal temperature, pH of growth, resistance to different concentrations of NaCl, investigation of catalase, arginine deaminize, gelatinize, lipase activities, casein and starch hydrolysis, reduction of nitrate to nitrite were done by the common well-known method. Anaerobic growth of the isolates was tested by growth in solid anaerobic stab cultures covered with paraffin layer. All mentioned experiments were carried out according to the technique described in [9, 10]. All isolated LAB were grown in skim milk with 0.1% methylene blue and in MRS broth with inverted Durham for studying the reductive activity of strains and gas-formation (homo- or hetero-fermentation activity). The basal medium with different carbohydrates was used to determine the LAB ability to produce gas and acid. API 50CH test ("Biomerieux", France) was used for identification of 4 isolated LAB.

DNA Typing Methods. Chromosomal DNA was isolated from single-colony inoculated 5 *mL* overnight cultures with GenEluteTM Bacterial Genomic DNA Kit ("Sigma Aldrich Co", USA). All primers and their applications are enumerated in Table.

Primers used in the investigation

№	Primer	5'→3' sequence	Annealing temperature, °*C*	Amplification product size	Target genes	Reference
16S rRNA genes sequencing						
1	fD1	AGAGTTTGATCCTGGCTCAG	56	1500 bp	16S rRNA	
2	rD1	TAAGGAGGTGATCCAGGC				
Genus-specific PCR						
3	Str A	AGAGTTTGATCCTGGCTCAG	35	500 bp	16S rRNA	[11]
4	Str B	GTACCGTCACAGTATGAACTTTCC	35			
5	Str I	TGTTTAGTTTTGAGAGGTCTTG	35	241 bp	16S-23S rRNA ISR	[12]
6	Str II	CGTGGAATTTGATATAGATAT	35			
7	P4 (S)	GGAGTTGGTGCTGGTATTGTG	35	497 bp	citrate permease	[13]
8	P5 (A)	CCAACCCTGCTGTAATAGCAG	35			
9	P6 (S)	CAACACGGCATGCATGTTGC	35	393 bp	protease	[14]
10	P7 (A)	CTGGCGTTCCCACCATTCA	35			
11	Ent 1	TACTGACAAACCATTCATGATG	35	112 bp	*tuf* seq. of *E. coli*	[15]
12	Ent 2	AACTTCGTCACCAACGCGAAC	35			
RAPD-PCR						
13	L2	ATGTAACGCC	35	different	n.a.	[15]
ERIC-PCR						
14	Eric 1r	ATGTAAGCTCCTGGGGATTCA	35	different	n.a.	[16]
15	Eric 2	AAGTAAGTGACTGGGGTGAGC	35			

If not mentioned otherwise all PCR reactions were performed in a final volume of 50 *μL* in a KCl buffer system containing 2.0 *mM* $MgCl_2$, 0.2 *mM* of each dNTP, 0.2 *mM* of both primers, 1 unit of Taq-polymerase ("Fermentas") at final concentrations, as well 40 *ng* of template DNA in total. After an initial denaturation at 94°*C* for 5 *min*, followed 35 cycles of denaturation at 94°*C* for 60 *s*, annealing temperature as mentioned in Table for 60 *s* and extension at 72°*C* for 30 *s*. A final extension step was performed during 5 *min* at 72°*C*. The amplification products were analyzed by applying 10 *μL* of the reaction mixture on 1.2% agarose gels visualized with ethidium bromide in the presence of 100 bp Ladder ("Fermentas").

Extracted DNA was used for random amplification of polymorphic DNA (RAPD), enterobacterial repetitive intergenic consensus (ERIC) and genus specified (GS) PCR using the primers listed in Table. As a positive control the DNA of referent strains *Streptococcus thermophilus* VKPM B3850 and *Enterococcus durans* ("BIA INRA", France) were used.

DNA Sequencing. Amplicons of DNA were sequenced using Macrogen (Republic of Korea). Homology searches were performed using the Blast algorithm [17] at the NCBI (National Center for Biotechnology Information: http://www.ncbi.nlm.nih.gov/Blast). All sequences were compared with other sequences in the GenBank database to identify closest relatives.

Phylogenetic Studies. Phylogenetic tree based on 16S rRNA gene sequences was made by DNASTAR and 452 ClastalX2 computer software. Phylogenetic analyses of the results of RAPD and ERIC-PCR were performed using GeneTools software (Syngene), and UPGMA dendrograms were constructed on the basis of the obtained similarity matrices.

Results and Discussion. The identification of LAB was based on routine physiological and biochemical tests, but as many features are inconsistent within individual species, the comparison with phylogenetic methods can rectify the obtained results. The identification based only on polyvalent investigation can give the most exact results, than the identification limited only by several unreliable key features.

Eight LAB strains isolated from matsun and cheese were selected, because they showed the highest antibacterial activity [18] and were used for future identification. The names of these strains were composed from capital letter of authors' names and serial number of dairy product sample, from which this strain had been isolated. As the best Armenian probiotic strain is indicator, which is used since 1971 for correction of dysbiosis in children, the *L. acidophilus* strain (Er 317/407), supplied by "Vitamax-E" LLC (Yerevan, Armenia) was used.

All studied strains were gram-positive, immotile and rod shaped. The exception was coccal INR-2010-Tsov-G strain. Optimal temperature of growth for all strains was determined to be between 37 and 40º*C*.

The growth pH range varied from 4 to 8. The optimum pH was 6. However some of the strains (Er 317/407, RIN-2003, INRA-2010-4.2, INA-5.1, INA-21.1 and INR-2010-Tsov-G) were growing also at pH 3, while others (RIN-2003, INA-5.1, INA-21.1 and INR-2010-Tsov-G) were growing even at pH 10. All strains grew at 0.5% NaCl concentration. Only few of them (INRA-2010-4.2, INA-5.1, INA-21.1 and INR-2010-Tsov-G) tolerated 2% NaCl concentrations. All strains were able to grow in anaerobic condition as well. All isolates were homofermentative, chemoorganotroph and catalase negative, none of them produced gas in the media containing glucose. They did not reduce nitrates to nitrites and did not hydrolyze gelatin and starch.

The ability to assimilate different carbohydrates is the most important point for LAB identification. Following the criteria of *Bergey's Manual of Determinative Bacteriology* [10], the isolates were identified as *Lactobacillus rhamnosus* (R-2002, INA-5.1, INA-21.1), *L. delbrueckii* subsp. *bulgaricus* (BAM-2003), *L. salivarius* (RIN-2003), *L. plantarum* (INRA-2010-4.2), *Streptococcus thermophilus* (INR-2010-Tsov-G), *L. helveticus* (INRA-2010-H11).

The identification of all strains was confirmed by 16S rDNA sequence analysis. The results from the phenotypic determination and 16S rRNA genes sequencing coincided with most of the strains (R-2002, INA-5.1, INA-21.1, BAM-2003, INRA-2010-H11). But for strains INRA-2010-4.2, RIN-2003, INR-2010-Tsov-G 16S rDNA analysis results differed from identification by phenotyping and, hence, they may be considered as new intermediate LAB strains.

According to the obtained results, the strain INR-2010-Tsov-G can grow at 55º*C* and the maximal concentration of NaCl in media should be 2%. Both these properties are typical for *Streptococci* strains, especially for *S. thermophilus*. However, the molecular genetic analyses revealed the belonging of this strain to species *Enterococcus durans* HM058837. Similarly, the strain RIN-2003 has been identified as *L. salivarius* according to fermentation of carbon sources, but multiple molecular genetic analyses revealed the identity of this strain with *L. delbrueckii* subs. *lactis* AB680073.

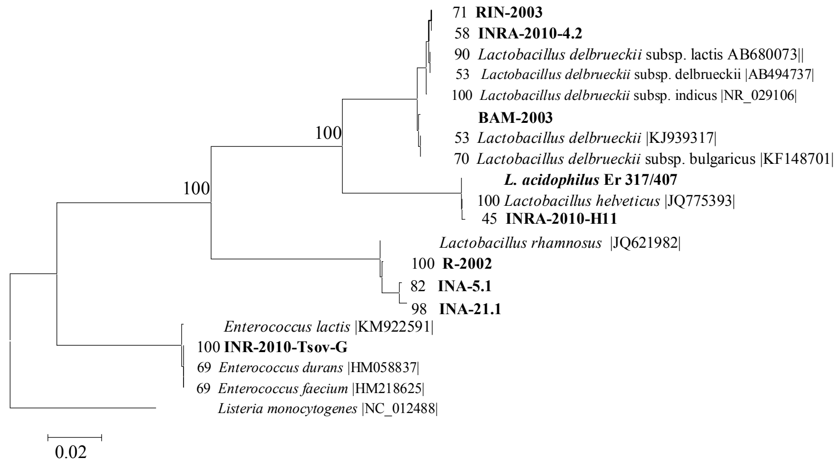

Fig. 1. Phylogenetic tree of isolates based on their 16S rDNA comparison.

The most interesting data are connected with control strain *L. acidophilus* Er 317/407. A phylogenetic tree including isolates and control species is presented in Fig. 1. All strains are divided into five main clusters, two of them are closer to each other. However, the strain INR-2010-Tsov-G was more thermophile than the other LAB and could not grow at high concentration of NaCl. Belonging of this strain to Enterococcus genera was also established by GS-PCR analyses (Fig. 2).

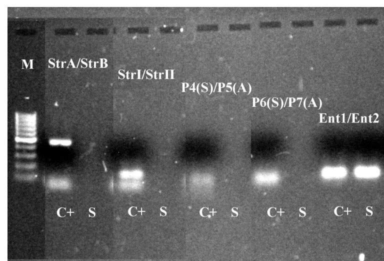

Fig. 2. Results of GS-PCR.

The genetic distance between strains BAM-2003, INRA-2010-4.2 and RIN-2003 can be seen from the results of RAPD-PCR presented in the Fig. 3.

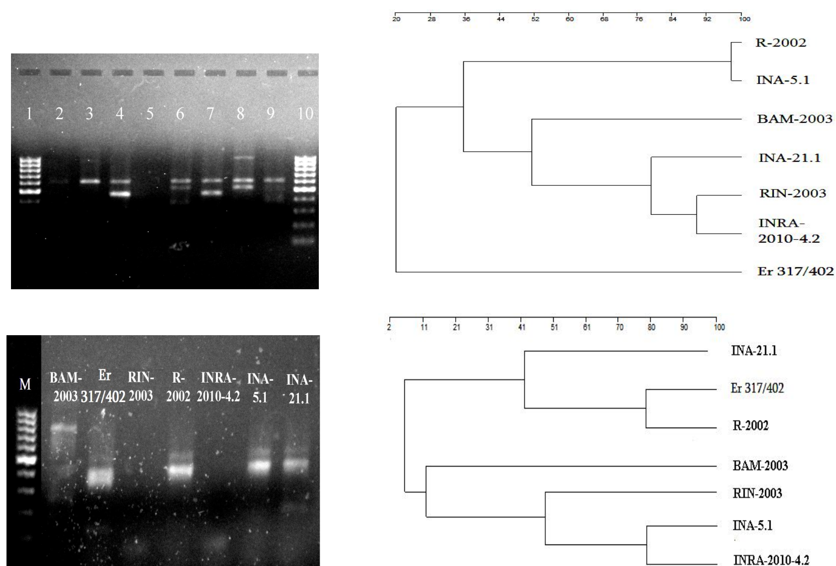

Fig. 3. Results of RAPD-PCR and ERIC-PCR.

As described in [19], biochemical properties and DNA-DNA re-association studies of *Lactobacillus acidophilus* strains isolated from humans and animals indicate that they include six species. Two new species can be differentiated from the established species of the genus Lactobacillus: *L. gallinarum* sp. nov. (type strain ATCC 33199) and *L. johnsonii* sp. nov. (type strain ATCC 33200). It was clarified that *L. acidophilus* group A3 [7] is synonymous with *L. amylovorus*. Taken together, these phenotypic data support the observation that sugar utilization in API tests is insufficient for the exact identification of closely related lactobacilli [20, 21].

All isolated and studied strains are conserved in the culture collection of the Department of Microbiology and Biotechnology of YSU. The strain R-2002 was identified as *L. rhamnosus* R-2002, according to the phenotyping characteristics, as well as 16S rRNA gene analysis (the sequence of 16S rRNA gene has been submitted to GenBank at the accession number KY054594) [22].

Conclusion. According to multiphase approach method all isolated LAB strains were identified: R-2002, INA-5.1, INA-21.1 – *Lactobacillus rhamnosus*, INRA-2010-4.2 and RIN-2003 – *L. delbrueckii* subs. *lactis*, BAM-2003 – *L. delbrueckii* subs. *bulgaricus*, INRA-2010-H11 – *L. helveticus*, INR-2010-Tsov-G – *Enterococcus faecium*. The studied LAB can be used as starter cultures not only for production of cheeses and matsuns, but for construction of new probiotics also.

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