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ISOLATION AND IDENTIFICATION OF LACTIC ACID BACTERIA FROM SOME AZERBAIJANI YOGURTS

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Isolation of lactic acid bacteria (LAB) was performed from three types of yogurts (Karabakh, Ganja, Baku). These products were produced in the territory of Azerbaijan. Karabakh and Baku yogurts are made from cow milk and Ganja yogurt - from buffalo milk. All types of yogurts were produced without the addition of lactic acid bacteria starter cultures. From these dairy products overall 178 isolates were isolated and after catalase test, Gram staining and microscopic observation, 115 were chosen for further analyses. The selection of LAB isolates was based on their proteolytic and antimicrobial activity. Based on the identification of LAB isolates by biochemical tests and molecular methods it was determined that four strains of LAB were primarily present in three yogurt types: Lactobacillus delbrueckii subsp. lactis, Lactobacillus delbrueckii subsp. bulgaricus and cocci representatives to species Streptococcus thermophillus and Enterococcus faecium. It was determined that 5 enterococci out of 115 tested isolates, were antimicrobial compounds producers.

Keywords: Non-starter lactic acid bacteria (NSLAB); Yogurts (Karabakh, Ganja, Baku); rep-PCR; 16S rDNA; Antimicrobial activity.

1. Introduction

Lactic acid bacteria (LAB) are widespread in nature. Non-starter lactic acid bacteria (NSLAB) constitute complex microbial associations that are characterized by the occurrence of various species and many biotypes as a result of a number of selective conditions persisting during the manufacturing process and different ecological niches (1, 2, 16, 17). The NSLAB community is composed mostly of mesophilic lactobacilli such as *Lactobacillus paracasei*, *Lactobacillus plantarum*, and *Lactobacillus curvatus* (22, 23). Nevertheless, pediococci, micrococci and leuconostoc could also be present (3, 7, 25). Pedoclimatic characteristics and genetic autochthonous variations create an environment so specific that it would be extremely difficult to reproduce elsewhere (4). The NSLAB dominate the microflora of long-ripened cheese for most of its ripening period and they certainly have the potential to affect and contribute to the cheese maturation (8).

Usually, for the taxonomical identification of the microbial flora in cheese phenotypic, biochemical and physiological tests have normally been used. These tests are often time-consuming and not fully reliable. The development of PCR-based molecular techniques for the identification of bacterial species offers new perspectives in microbial taxonomic studies (3, 5, 9, 27).

The study of strain heterogeneity in natural cheese and yogurts starters is believed to be of great importance to the dairy industry (8, 11).

The aim of this study was isolation, characterization and identification of lactic acid bacteria by the molecular methods from three types of Azerbaijani yogurts. There are 9 climate zones in Azerbaijan which condition the presence of huge ecological diversities.

Immemorial pastoral traditions of many ethnic groups which were transiting or dwelling in this region were kept. They evolved a traditional way of different dairy products production, particularly cheeses and yogurts.

2. Materials and methods

The manufacture of all three types of yogurt is very similar and it has been done, throughout years, in the same traditional way. Karabakh and Baku yogurts are made from cow milk and Ganja yogurt from buffalo milk.

The production of yogurt is done in the following way: 10 ml of old yogurt is added in 1 liter of pasteurized milk chilled at 37 to 40 ºC. After the inoculation, the milk is stored at this temperature in glass bank or saucepan until the curd is formed (4 to 8 h). The pasteurization of milk is done by warming of milk to the 70 °C temperature during few minutes.

The samples of yogurts were obtained in May 2004. These samples were taken from farmhouses in a sterile plastic container and transported to the laboratory in a refrigerator.

For the isolation of bacteria from yoghurt, 10 ml of each sample was taken and homogenized with pastille in sterile mortar and transferred to 90 ml sterile 2% sodium citrate solution in a sterile chemical flask. Decimal dilutions of the homogenates were prepared with sterile 0.85% (w/v) sodium chloride and were plated on media most suitable for isolation of LAB. Lactic acid bacteria were isolated on MRS agar pH 5.7 (Merck, Darmstadt, Germany) and on M17 agar pH 7.2 (Merck, Darmstadt, Germany) after incubation at 30 °C and 45 °C for three days in aerobic and in anaerobic conditions (9). Incubation in anaerobic conditions was carried out using the anaerobic jars with Anaerocult A (Merck, Darmstadt, Germany). Total counts of bacteria were determined and the mean of the triplicate plates was noted. Results were expressed as colony forming units (CFU) per milliliter of yogurt.

The LAB was isolated from three samples of yoghurt. Samples were designnated AZEJ1 – yogurt Karabakh, AZEJ2 – yogurt Ganja and AZEJ3 – yogurt Baku.

Fifty to one hundred colonies per yogurt sample were randomly taken from both MRS and M17 (30^{o}C and 45^{o}C) agar plates corresponding to the highest dilution at which growth occurred. Cell morphology of all strains of LAB was determined in a microscope (Olympus U-RFL-T, BX51, GmbH, Hamburg, Germany). After microscopic observations, the colonies were sub-cultured to purity on MRS or M17 medium. Gram positive and catalase-negative isolates were stored at –20^{o}C and –80^{o}C in M17 (for cocci) and in MRS (for rods) broth containing 15% of glycerol (v/v) (23). Overall 178 isolates were isolated and after catalase test, Gram staining and microscopic observation, 115 were chosen for further analyses.

For 23 isolates of LAB, which were chosen upon the analysis of their proteolytic activity on ß-casein, plasmid profiles and identification by rep-PCR method, further characterization and tests followed: (a) growth at 30 and 45 ^{o}C in MRS and M17 broth, (b) salt tolerance - 4.0 and 6.5% (w/w) NaCl in MRS and M17 broth, (c)

production of carbon dioxide from glucose by sub-culturing the isolates in tubes with MRS broth Durham's tubes, (d) L-arginine hydrolysis, (e) activity in milk (for all 296 isolates), (f) black zone formation on esculin bile agar (Himedia, Mumbai, India), done only for coccoid shapes of LAB. Identification of the isolates was performed according to the methods and criteria of Sharpe, 1979; Hardie, 1986; Kandler and Weiss, 1986; Mundt, 1986a,b; Sneath et al., 1986 (18,20,26,27, 32,33). The tests (a) and (b) are performed three times.

The fermentation of carbohydrates was determined on MRS broth containing bromcresolpurple (0,04 g l^{-1}) as a pH indicator. The carbon sources were added to the medium to give a final concentration of 1% (w/v). The carbohydrates tested were: L-arabinose, cellobiose, esculin, fructose, galactose, glucose, glycerol, inuline, lactose, maltose, melibiose, mannitol, mannose, raffinose, rhamnose, ribose, salicin, sorbose, sorbitol, starch, saccharose, trehalose and D-xylose.

Antimicrobial activity of isolated lactic acid bacteria was screened by agar-well diffusion method (31) using *Lactobacillus bulgaricus 340* as indicator strain. Soft MRS agar (0.7%, w/v), containing indicator strain, was overlaid onto MRS plates. Wells were made in the lawn of hardened soft agars. Aliquots (50µl) of supernatant of overnight cultures (16 h) were placed in the wells. To confirm the production of proteinaceous substance, a crystal of proteolytic enzyme pronase E (Sigma Chemie GmbH, Deisenhofen, Germany) was placed close to the edge of supernatant containing well. The plates were incubated overnight at 30 °C. A clear zone of inhibition around the well, but not in the vicinity of pronase E crystal, was taken as an indication of proteinaceous nature of produced antimicrobial substance i.e. a potential bacteriocin-like compound.

Proteolytic activities of the isolates were assayed as previously described (17). Collected fresh cells (10 mg approximate density 10^{10} cells ml^{-1}) were resuspended in 0.1 mol l^{-1} sodium-phosphate buffer, pH 6.5. The cell suspension was mixed with β-casein (5 mg ml^{-1} in 0.1 mol l^{-1} sodium-phosphate buffer, pH 6.5) (Sigma, St. Louis, MO, USA) and incubated 3 h at 30 °C. Electrophoresis was carried out on 12.5% polyacrylamide gel. Gels were stained with Coomassie brilliant blue G250 (SERVA, Heidelberg, Germany) and destained in a mix of methanol (20%) and acetic acid (7%).

A total DNA from cheese was extracted as described by Randazzo et al.(2001) (31), and a total DNA from LAB isolates was purified by the method given by Hopwood et al. (1985) (21).

All PCR amplifications were performed with the *Taq* DNA polymerase kit (Fermentas UAB, Vilnius, Lithuania). Reaction mixtures consisted of 0.02 mol l^{-1} Tris-HCl (pH 8.4), 0.05 mol l^{-1} KCl, 0.003 mol l^{-1} $MgCl_2$, 0.05 mol l^{-1} each of the four deoxynucleotide triphosphates (dNTP), 1 U of *Taq* polymerase, 5 pmol l^{-1} of each primer, and 1 µl of template DNA in a final volume of 50 µl.

For the repetitive extragenic palindromic polymerase chain reaction (rep-PCR) analysis total DNA from different isolates of LAB was used as template for PCR amplifications with BOXA1R (5′-CTACGGCAAGGCGACGCTGACG-3′) and $(GTG)_5$ (5′-GTGGTGGTGGTGGTG-3′) oligonucleotide primers, each with its optimal PCR program (38), using Taq DNA polymerase (Fermentas UAB, Vilnius, Lithuania). Reactions were carried out in a thermal cycler Gene Amp^R PCR System 2700 (Applied

Biosystems, Foster City, CA, USA). The PCR products were separated by electrophoresis on 1.5% agarose gel (15 x 20 cm) containing 0.5 μg ml^{-1} ethidium bromide, for 16 h in 1 x TAE buffer (40 mmol l^{-1} Tris acetate, 1 mmol l^{-1} EDTA) and 55 V (constant voltage) at 4 °C (39). Electrophoresis was performed using an Electrophoresis Power Supply - EPS 301 (Amersham Biosciences, Piscataway, New Jersey, USA). The rep-PCR profiles were visualized under ultraviolet light, followed by digital image capturing using a CCD camera Biometra BDR2/5/6 (Bio Doc Analyze GmbH, Göttingen, Germany).

For the sequencing of the 16S rRNA region total DNA was used as a template for PCR amplifications with U968 (5´-AACGCGAAGAACCTTAC-3´) and L1401 (5´-GCGTGTGTACA AGACCC-3´) primers (30,33), using Taq DNA polymerase (Fermentas UAB, Vilnius, Lithuania). Reactions were carried out in thermal cycler as cited above. Obtained PCR product was purified by QIAquick PCR Purification KIT/250 (QIAGEN GmbH, Hilden, Germany), and sequenced by CRIBI-BMR servizio sequenziamento DNA, Univesita di Padova, Italy. The sequence was aligned in the NCBI database using the standard nucleotide-nucletide homology search BLAST (The Basic Local Alignment Search Tool) (http: // www. ncbi. nlm. nih. Gov / BLAST).

3. Results and discussion

From all yogurt samples used in the study, 178 randomly chosen isolates of LAB were taken for the analysis. The results showed that 115 isolates were gram positive and catalase negative and they were taken for further examination of proteolytic activity, activity in milk and production of antimicrobial compounds.

Yogurt samples were poor with bacterial colonies on the petri dishes with both MRS an M17 medium, on which a significant number of yeasts (1.9 x 10^6 CFU ml^{-1} for AZEJ1, 1.8 x 10^5 CFU ml^{-1} for AZEJ2 and 1.4 x 10^6 CFU ml^{-1} for AZEJ3) and moulds (4 x 10^4 CFU ml^{-1} for AZEJ2 and 3 x 10^4 CFU ml^{-1}) were grown. From a total of 70 isolates of Karabakh yogurt, 52 were gram positive and catalase negative. From a total of 55 isolates from Ganja yogurt, 33 were gram positive and catalase negative, and from Baku yogurt 30 gram positive and catalase negative LAB were isolated from a total of 53 isolates. A large number of cocci formed black zone on esculin bile agar, so it is more likely that they were enterococci.

The results of viable counts of bacteria on MRS and M17 medium in Karabakh, Ganja and Baku yogurts are summarized in Table 1.

Most variations of total viable counts were found in the Karabakh yogurt. In this yogurt the total viable counts of bacteria on MRS medium were between 1.49 x 10^4 CFU ml^{-1} and 1.55 x 10^4 CFU ml^{-1}, and on M17 medium from 7.24 x 10^7 CFU ml^{-1} to 3.86 x 10^8 CFU ml^{-1}.

Table 1

Mean number of total viable bacteria in samples of different Azerbaijani yogurts on MRS and M17 medium

Sample	CFU/ml sample[a]			
	MRS at 30 °C	MRS at 45 °C	M17 at 30 °C	M17 at 45°C
AZEJ 1	1.55 x 10^4	1.49 x 10^4	7.24 x 10^7	3.86 x 10^8
AZEJ 2	4.02 x 10^6	3.04 x 10^7	3.96 x 10^6	3.58 x 10^7
AZEJ 3	9.20 x 10^5	2.16 x 10^7	2.58 x 10^6	1.78 x 10^7

[a] Average values of three repetition.

Also, the results from Table 1 showed that thermophilic microflora on MRS and M17 mediums was more numerous than mesophilic microflora on the same mediums. The only exception was total viable count of thermophilic bacteria on MRS medium (45 °C) in sample of Karabakh yogurt (1.49 x 10^4 CFU ml^{-1}) which was lesser then the total count of mesophilic bacteria on MRS medium (30°C) which was 1.55 x 10^4 CFU ml^{-1}.

Activity in milk was tested for all of 115 isolates of LAB isolated from Karabakh, Ganja and Baku yogurts. 7 streptococci isolates (AZEJ3-27, AZEJ3-28, AZEJ3-29, AZEJ3-30, AZEJ3-31, AZEJ3-38 and AZEJ3-43), exhibited good acidification activity in milk, and after 4.5-6 h of incubation the pH reached a value of 4.8. Most of LAB isolates from Ganja and Baku yogurts form the curd in skimmed milk during 16, 24 and 48 hours (results not shown).

Proteolytic activity, activity in milk and antimicrobial activity were examined on all 115 gram positive and catalase negative LAB isolates, isolated from different types of Azerbaijani yogurts. Based on those results 23 LAB isolates have been made for rep-PCR analysis.

According to the results of rep -PCR, analysis of some phenotypic characteristics (growth at 30 and 45 °C, growth in bouillon with 4 and 6,5% of salt concentration, ability of arginine hydrolysis and production of CO_2 from glucose), and fermentation of already mentioned carbohydrates, 23 LAB isolates were chosen. The results of phenotypic characterization of LAB were shown in Table 2. The LAB identification based on this analysis, has shown that, most of the lactobacilli from AZEJ1, AZEJ2 and AZEJ3 yogurt samples belonged to species *Lactobacillus delbrueckii* subsp. *lactis*, while according to phenotypic characterization, the largest number of cocci belonged to species *Enterococcus faecium* and *Streptococcus thermophilus.*

Table 2

Some phenotype characteristics and carbohydrate patterns among LAB isolates from samples of different kinds of Azerbaijani yogurts

Tests	Species											
	1	2	3	4	5	6	7	8	9	10	11	12
Growth at 30°C	+11	+	+	+	+	+	+	+	+	+	+	+
Growth at 45°C	-	+	+	+	+	+	+	-	+	+	+	+
Growth at 4%NaCl	+	-	-	-	-	-	+	-	+	+	+	+
Growth at 6%NaCl	+	-	-	-	-	-	+	-	+	+	+	+
Hydrolysis of a r a arginine	+	±	±	+	+	+	-	±	+	+	+	+
Prod. of CO_2 f from glucose	+	-	-	-	-	-	+	-	-	-	-	-
Glycerol	-	-	-	-	-	-	-	-	-	-	-	-
L-arabinose	+	+	+	+	±	+	-	-	+	+	+	+
Ribose	+	±	+	+	+	+	±	-	+	+	+	+
D-xylose	+	±	+	+	+	+	-	-	-	-	-	-
Galactose	-	+	+	+	+	+	±	-	+	+	+	+
Glucose	+	+	+	+	+	±	+	+	+	+	+	+
Fructose	+	+	+	+	+	+	+	-	+	+	+	+
Mannose	+	+	+	+	+	+	+	-	+	+	+	+
Sorbose	-	-	-	-	-	-	-	-	-	-	-	-
Rhamnose	-	-	-	-	-	-	-	-	-	-	-	-
Mannitol	-	+	+	+	+	+	-	-	-	-	-	-
Sorbitol	+	-	-	-	-	-	-	-	-	-	-	-
Esculin	+	+	+	+	+	+	-	-	+	+	+	+
Salicin	+	+	+	±	+	+	-	-	±	±	±	±
Cellobiose	+	±	-	-	-	-	-	-	+	+	+	+

Maltose	+	+	+	+	+	+	-	-	+	+	+	+
Lactose	+	+	+	+	+	+	+	+	+	+	+	+
Melibiose	+	-	-	-	-	-	-	-	+	+	+	+
Saccharose	+	+	+	+	+	+	+	-	-	-	+	+
Trehalose	+	+	+	+	+	+	-	-	±	±	+	±
Inuline	-	-	-	±	-	-	-	-	-	-	-	-
Raffinose	+	±	±	±	±	-	±	-	-	-	-	-
Starch	-	-	-	-	-	-	-	-	-	-	-	-

+: positive reaction; -: negative reaction; ±: weak reaction; 1: *Lactobacillus brevis* (AZEJ2-77); 2-6: *Lactobacillus delbrueckii* subsp. *lactis* (AZEJ3-92, AZEJ1-49, AZEJ1-56, AZEJ1-62, AZEJ3-76), 7: *Lactobacillus delbrueckii* (AZEJ2-95); 8: *Streptococcus thermophilus* (AZEJ1-35); 9-12: *Enterococcus faecium* (AZEJ3-34, AZEJ2-2, AZEJ3-27, AZEJ3-29, AZEJ2-43, AZEJ3-43, AZEJ2-41, AZEJ2-3).

For all 115 isolates of LAB from different kinds of samples of Azerbaijani dairy products proteolytic activity was analyzed. Examination of proteolytic activity revealed that 8 lactobacilli, 9 lactococci and 1 isolate of enterococci from Karabakh yogurt , 9 lactobacilli from Ganja yogurt and 2 isolated lactobacilli from Baku yogurt exhibited a strong proteolytic activity (results not shown). A small number of lactobacilli (5.7%) and of lactococci (7.7%) isolated from all yogurt samples showed a good proteolytic activity, while the rest of lactobacilli and lactococci, including also the large number of enterococci degraded ß-casein poorly, or did not degrade it at all (results not shown).

It was confirmed that only 5 out of 115 analyzed LAB isolates produced antimicrobial compounds. Experiments with pronase E revealed a proteinous nature of antimicrobial compounds, indicating the possibility they could be bacteriocin-like substances. One of antimicrobial compounds producers were isolated from Karabakh yogurt, two isolate- from Baku yogurt and two producer of antimicrobial compounds were isolated from Ganja yogurt. Isolates exhibited a clear or turbid zone of inhibition. Isolate AZEJ1-48 gave zone of inhibition on 5 indicator strains, isolates AZEJ2-34 and AZEJ3-30 gave zone of inhibition on 2, AZEJ2-34 – on 3 indicator strains, while isolate AZEJ3-48 gave zone of inhibition on 4 indicator strains from a total of 10 indicator strains used in the test (Table 3). It was identified by rep-PCR and 16S rDNA sequencing that the all isolates which produced antimicrobial compounds belonged to the species *Enterococcus faecium*.

Table 3

Antimicrobial activity of LAB from samples of yogurts AZEJ1, AZEJ2 and AZEJ3

Indicator strains	AZEJ1-48	AZEJ2-34	AZEJ2-41	AZEJ3-30	AZEJ3-48
BGMN1-5	Clear zone		Clear zone		Turbid zone
BGMN1-596	Clear zone				
S50			Turbid zone		
NS1		Clear zone		Clear zone	
BGBUK2-8	Clear zone				Clear zone
BGBUK2-16	Turbid zone	Turbid zone			Turbid zone
A112	Clear zone				
BGK4			Turbid zone		
BGLI15					Turbid zone
BGKP20				Turbid zone	

According to the proteolytic activity of LAB, 23 rods and cocci were chosen for molecular identification with rep-PCR (Fig. 1 and Fig. 2). The results revealed

that 2 of 7 chosen lactobacilli were identified as *Lactobacillus para plantarum* and 1 as *Lactobacillus plantarum.* 4 isolates of lactobacilli were not identified by this method (AZEJ1-49, AZEJ1-62, AZEJ2-96 and AZEJ3-76). Basing on the same method 16 cocci were identified as follows: 7 belonged to the species *Enterococcus faecium,* 5 were identified as *Streptococcus thermophilus* and 4 isolates of cocci were not identified by the rep-PCR method (AZEJ1-35, AZEJ2-43, AZEJ2-48 and AZEJ1-56).

Table 4

Isolates of LAB identified by rep-PCR and 16S rDNA sequencing

Isolate	Identification by rep-PCR	Identification by 16S rDNA Sequencing (% identity)
AZEJ1-35	Unidentified	*Enterococcus faecium* 100%
AZEJ1-48	*Enterococcus faecium*	*Enterococcus faecium* 99%
AZEJ1-49	Unidentified	*Lactobacillus delbrueckii* subsp. *lactis* 100%
AZEJ1-56	Unidentified	Not determined
AZEJ1-62	Unidentified	*Lactobacillus delbrueckii* subsp. *lactis* subsp. *bulgaricus*/subsp. *delbrueckii* 98%
AZEJ2-2	*Enterococcus faecium*	*Streptococcus thermophillus* 100%
AZEJ2-34	*Enterococcus faecium*	*Enterococcus faecium* 99%
AZEJ2-41	*Enterococcus faecium*	*Enterococcus faecium* 100%
AZEJ2-43	Not determined	*Enterococcus faecium* 100%
AZEJ2-48	Not determined	*Enterococcus faecium* 99%
AZEJ2-77	*Lactobacillus paraplantarum*	*Lactobacillus plantarum* 99%
AZEJ2-89	*Lactobacillus paraplantarum*	*Lactobacillus delbrueckii* subsp. *bulgaricus* 100%
AZEJ2-96	Unidentified	*Lactobacillus delbrueckii* subsp. *bulgaricus* 99%
AZEJ3-7	*Enterococcus faecium*	*Streptococcus thermophilus/Str. salivarius* 99%
AZEJ3-27	*Streptococcus thermophilus*	*Streptococcus thermophilus/Str. salivarius 99%*
AZEJ3-29	*Enterococcus faecium*	Not determined
AZEJ3-30	*Streptococcus thermophilus*	*Streptococcus thermophilus/Str. salivarius* 99%
AZEJ3-31	*Enterococcus faecium*	Not determined
AZEJ3-32/2	*Lactobacillus plantarum*	Not determined
AZEJ3-36	*Streptococcus thermophilus*	Not determined
AZEJ3-43	*Streptococcus thermophilus*	Not determined
AZEJ3-48	*Streptococcus thermophilus*	*Enterococcus durans/Ent. avium/Ent. carnosus* 99%
AZEJ3-76	Unidentified	*Lactobacillus delbrueckii* subsp. *bulgaricus/ subsp. lactis/* subsp. *delbrueckii* 99%

There are huge discrepancies in the LAB identification results obtained from phenotypic characterization, the ability of carbohydrate fermentation and by rep-PCR method. Subsequent final identification of these isolates (23 isolates of LAB) was done according to 16S rDNA sequencing (Table 4). The results of sequencing showed that 4 of 7 chosen lactobacilli were identified as *Lactobacillus delbruckii*, 1 as *Lacto-*

bacillus plantarum. Basing on the same method 16 cocci were identified as follows: 6 belonged to the species *Enterococcus faecium,* 3 were identified as *Streptococcus thermophilus/Streptococcus salivarius* and 2 isolates of cocci (AZEJ3-48 and AZEJ2-2) were identified as *Enterococcus durans/Enterococcus faecium / Enterococcus carnosus* and *Streptococcus thermophilus*, respectively.

The process of dairymaking has begun about 8000 years ago and now only more than 1000 cheese varieties exist world wide (35), each unique with respect to its flavor and form. The manufacture of most cheese varieties involves combining of four ingredients: milk, rennet, microorganisms and salt (3). The ripening process is probably the most significant period of cheese production, during which starter and nonstarter bacteria, chymosin, and the indigenous milk enzymes develop the organoleptic and textural properties of the cheese (4).

The Karabakh, Baku and Ganja yogurts are dairy products from Azerbaijan, the region of specific geographic location and climate zone, still produced in a traditional way. Therefore, there was a huge interest for studying the lactic acid bacteria in these dairy products, particularly since no scientific data regarding these dairy products exist.

Before the isolation of the lactic acid bacteria, the total number of bacteria was determined, and in all three types of yogurt time the total of bacteria ranged between 10^4 and 10^8 CFU g^{-1}. It was lower than them values corresponding to those usually found in cheeses during ripening number (11, 12).

Regarding the bacteria composition in some Azerbaijan dairy products, it was ascertained that lactobacilli, lactococci and enterococci were present in yogurts. Enterococci are recognized as an essential part of the natural microflora of many dairy products and, in some cheeses, they dominate over lactobacilli and lactococci (4, 5, 19, 35, 36, 37). The reasons for the prevalence of enterococci in dairy products have long been considered to be the result of unhygienic conditions during collection and processing of milk, together with their resistance to pasteurization temperatures and their adaptability to different substrates and growth conditions (10). Enterococci have been reported to be one of the most resistant microbiological groups to adverse conditions such as salt and acidity, which explains their predominance in some cheeses (7).

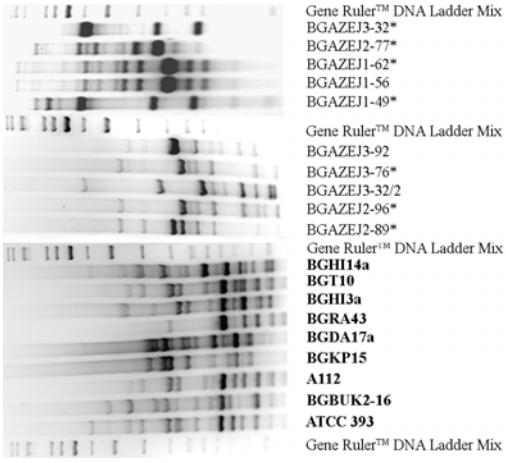

Fig. 1. The BOXA1R rep-PCR analysis of lactobacilli isolated from different kinds of Azerbaijani dairy products. Reference strains used in the test are given in bold letters.

* Isolates also identified by 16S rDNA sequencing.

The analysis of the proteolytic activity revealed that relatively small number (about 20%) of lactobacilli and lactococci, isolated from all 3 types of Azerbaijan products, showed good ability of β casein degradation. Numerous enterococci present in these products showed very low or no proteolytic activity. By different methods of identification used in this research, they were identified as *Enterococcus faecium.* In a recent investigation (24,28,29,34) it has been noted that *Enterococcus faecalis* strains showed generally better performance compared to *Enterococcus faecium* and *Enterococcus durans,* in respect to some biochemical properties such as the acidifying ability and proteolytic activity.

Phenotypic characterization and carbohydrates fermentation of for LAB isolated from all three yogurt types (Karabakh, Ganja and Baku) revealed that lactobacilli belonged to the species *Lactobacillus delbrueckii* subsp. *lactis,* and cocci representatives to species *Streptococcus thermophillus* and *Enterococcus faecium.* However, this identification is based only on classical microbiological methods and authentic identity of species should be confirmed by molecular identification.

Using the rep-PCR method it was established that in yogurts *Streptococcus thermophilus* and *Enterococcus faecium* were present. A certain number of lactic acid bacteria isolates (mostly lactobacilli isolated from yogurt samples) was not identified by this method. Besides that, some yogurt cocci isolates, identified as *Streptococcus thermophillus* based on phenotypic characterization and the fermentation of carbohydrates, were identified as *Enterococcus faecium* by the rep-PCR method and vice versa. Corroler et al. (1998) reported that natural lactococci can show atypical phenotypic characterization (for example: grow in presence of 6,5% NaCl) and commonly survive in hostile condition. Additionally, the phenotypic characterization highlighted a high degree of variability in *Streptococcus thermophilus* despite the analyzed strains being principally isolated from yogurt samples. As recently reported by Giraffa et al. (2001) and Mora et al. (2002) some strains were able to ferment galactose, suggesting that this phenotypic trait should be considered as variable in the taxonomic description of the species *Streptococcus thermophilus* (6,13,14,15,26).

The difficulties in the identification of some isolates by rep-PCR method occurred because of the interspecies differences of band patterns which are most probably the result of genetic variability within the species. Because of the dismatch of the lactic acid bacteria identification results, large number of isolates (23 of them) were identified by 16S rDNA sequencing. In that occasion it was confirmed that in the samples of all three yogurt types (Karabakh, Ganja and Baku) the species *Lactobacillus delbrueckii* subsp. *lactis*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Streptococcus thermophilus* and *Enterococcus faecium* were identified by 16S rDNA sequencing.

The largest number of enterococci (24 isolates total) was isolated from yogurts. Although the genus *Enterococcus* comprises the most controversial group of LAB, the contribution of enterococci to the organoleptic properties of fermented foodstufs (cheese, sausages, vegetables and olives) and also their ability to produce enterocins are important characteristics for their application in food technology (5,6,8).

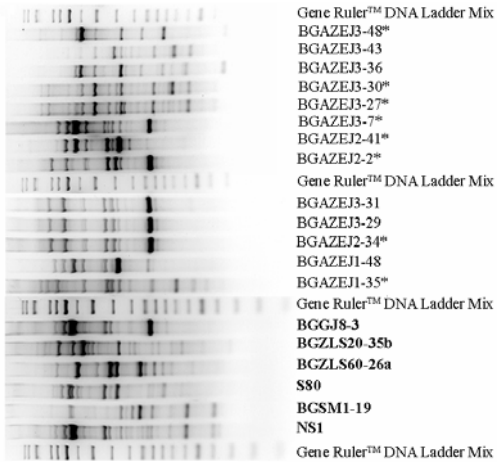

Figure 2. The $(GTG)_5$ rep-PCR analysis of cocci isolated from different kinds of Azerbaijani dairy products. Reference strains used in the test are given in bold letters.

- Isolates also identified by 16S rDNA sequencing.

Overall, 5 coccoids isolated from all types of yogurts, identified as *Enterococcus faecium* (AZEJ1-48 AZEJ2-34, AZEJ2-41), *Streptococcus thermophilus* (AZEJ3-30) and *Enterococcus durans* (AZEJ3-48), showed antimicrobial activity and created inhibition zones on 2, 3, 4 or 5 different indicator strains. Strains of enterococci , including *Enterococcus faecium, Enterococcus durans* and *Enterococcus faecalis,* are known to produce bactericidal peptides, which are called enterocins, that generally belong to class II bacteriocins (9). The enterocins are usually active against other enterococci, lactic acid bacteria, *Listeria monocytogenes*, *Staphylococcus aureus*, and *Clostridium* spp., including *Clostridium botulinum, Clostridium perfringens* and *Clostridium tyrobutyricum* (9). For further elucidation of the nature and activity of these antimicrobial compounds produced by enterococci from Azerbaijani cheese and yogurt, more detailed analysis is required.

In the present study, both phenotypic and genotypic approaches were used to identify LAB in different kinds of Azerbaijani dairy products. The experiments performed in our study demonstrated that the yogurts of Karabakh, Ganja and Sheki were rich with species *Lactobacillus delbrueckii* subsp. *lactis*, *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*. The species *Enterococcus faecium* and *Enterococcus durans* were also present. More detailed research of lactic acid bacteria was done on one sample of each product, and it would be necessary to do the examination of lactic acid bacteria on more samples of these products. In addition, in order to improve Azerbaijani dairy products further study of technological properties of selected strains will be evaluated in pilot-scale yogurt production. Those isolates showing good proteolytic activity, the ability of lactic acid production in a short period of time, which produce antimicrobial compounds will be included in this study. Our results are very promising and open up the possibility for further research of some LAB isolates that could constitute a starter culture for the industrial production of these types of Azerbaijani yogurts.

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AZƏRBAYCANIN BƏZİ QATIQ NÖVLƏRİNDƏN SÜD TURŞUSU BAKTERİYALARININ AYRILMASI VƏ İDENTİFİKASİYASI

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XÜLASƏ

Sud turşusu bakteriyaları Azərbaycanda istehsal edilən 3 qatıq (Qarabağ, Gəncə və Bakı) növündən izolə edilmişdir. Qarabağ və Bakı qatıqları inək südündən, Gəncə qatığı isə camış südündən hazırlanmışdır. Bu məhsullardan ayrılmış cəmi 178 koloniyadan 115-i Gram müsbət və katalaza-mənfi olmuşlar. STB-lərin seleksiyası onların proteolitik və antibakteriyal fəallıqlarına əsaslanmışdır. Bakteriyalar biokimyəvi və molekulyar metodlarla identifikasiya edilmişlər. Qatıq növlərində STB-lərin *Lactobacillus delbrueckii* subsp. *lactis, Lactobacillus delbrueckii* subsp. *bulgaricus, Streptococcus thermophilus* və *Enterococcus faecium* növləri aşkar edilmişdir.

ИЗОЛИРОВАНИЕ И ИДЕНТИФИКАЦИЯ МОЛОЧНОКИСЛЫХ БАКТЕРИЙ ИЗ НЕКОТОРЫХ ВИДОВ АЗЕРБАЙДЖАНСКИХ ЙОГУРТОВ (КАТЫК)

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РЕЗЮМЕ

МКБ были изолированы из 3 видов йогуртов - катыков (Карабах, Гянджа и Баку) возделываемых в Азербайджане. Йогурты Карабах и Баку были возделаны из коровьего молока, а Гянджа – из молока буйвола. Из всех изолированных 178 колоний 115 были Грам-положительными и каталаза-отрицательными. Скрининг МКБ был основан на их протеолитическим и антибактериальным активностями. Идентификация бактерий была осуществлена биохимическими и молекулярными методами. МКБ в йогуртах определены как *Lactobacillus delbrueckii* subsp. *lactis, Lactobacillus delbrueckii* subsp. *bulgaricus, Streptococcus thermophilus* и *Enterococcus faecium*.