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Biotechnology

Probiotic Properties and Biotechnological Potential of Lactic Acid Bacteria Isolated from the Diverce Areas of Georgia

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Despite the recent rapid expansion of the world of probiotics, the search for new starter cultures among lactic acid bacteria is actively underway. The main goal of our work was the isolation of lactic acid bacteria, *autochthonous* for Georgia and study of their probiotic and biotechnological potential, important for their application of fermented milk-beverage. The lactic acid bacteria were isolated from raw milk, spontaneously fermented raw milk, Matsoni, cheese whey and phyllosphere of plants collected in different regions of Georgia. A total of 263 isolates were obtained from 116 samples. Among them, 21 strains were identified as *L. delbrueckii* and 5 strains as *S. thermophilus* according to the 16S rDNA PCR products analysis. As a result of multi-step screening, based on probiotic, biotechnological, and organoleptic characteristics, 4 autochthonous strains were selected: *S. thermophilus* T-365, *S. thermophilus* G-26, *L. delbrueckii* subsp. *bulgaricus* T-190 and *L. delbrueckii* subsp. *lactis* T-221. These strains, either individually or in combination can be used as starters to produce Georgian topical fermented milk beverages. © *2024 Bull. Georg. Natl. Acad. Sci.*

lactic acid bacteria, fermented milk product, organoleptic properties

Recently, the demand for probiotic products has been growing worldwide with the increasing of their number by approximately 10% per year in the EU [1]. The global market for yogurt-like products reach USD 44.46 billion by 2025, with a compound annual growth rate (CAGR) of 4.8%. Of these, the largest market growth is expected to experience yogurt-type beverages compared to plant-based beverages. This is due to their greater health benefits and lower costs [2]. A wide range of probiotic products is available in the markets of various European countries, including cereal-based products [3], the fruit or vegetable juices [4, 5], although the majority of them are dairy products [6]. Representatives of bifidobacteria and various genera of lactic acid bacteria (LAB), as well as propionic acid bacteria, some species of enterobacteria, the genus *Bacillus* and yeasts are used for the production of probiotic foods [7]. LAB and bifidobacteria are representatives of the normal microbiota of the human digestive tract. They are the leading source of probiotics and most well-

studied microorganisms with a long tradition of safe use in the food industry [6]. Dairy propionic acid bacteria are used both for cheese ripening and as biopreservatives and useful additives in the food industry [8]. Starters are of great industrial importance for the efficient production of fermented milk products, improving their taste and consistency.

Despite the growing recognition of probiotics over the past few years, which has led to a rapid expansion of the probiotic world, the search for new starter cultures is still actual. Representatives of the genus *Lactobacillus* remain as the most wide spread source of probiotics [7]. The goal of the work is to isolate lactic acid bacteria from milk and fermented milk products, from the phyllosphere of various plants and study their properties with the aim of their potential application together with propionic acid bacteria for production of a Matsoni-like fermented milk beverage.

Methods and Results

LAB were isolated from raw milk, self-fermented raw milk, cheese whey and phyllosphere of plants collected in different regions of Georgia: raw milk and dairy products – 77 samples, including 30 milk samples (1 from goat milk, the others – cow milk), self-fermented raw milk – 33 samples, Matsoni – 14 (1 from buffalo milk, the others from cow's milk), from the whey remaining after cheese-making – 8; from the different aboveground parts of plants (hops, sorrel, lecir, chamomile, shindig, calendula, elderberry, Christ's blood, apple fruit, etc.) – 31. Milk and milk product samples, as well as plant samples for the isolation of lactic acid bacteria were collected in the following villages of different municipalities of Georgia: Chobareti, Khalilo, Alotsi, Javakhi, Dusheti, Sagarejo, Khviti, Nikozi, Ghari, Khvanchkara, Chala, Akhali Samgori, Korbouli, Tbilisi, Lagodekhi, Kveda Lukha, Chuberi, Okrokana, Sviri, Manglisi, Tskhrukveti, Kazreti, Tsalka, Tamarisi, Mukhuri, Khoni, Gordi, Kinchkhi and Vani.

Lactobacilli have been isolated on MRS agar medium at 37°C and *Streptococcus thermophilus* – on M17 agar medium at 42°C under anaerobic conditions for 48-72 h of cultivation. Pure cultures of the isolates were obtained by repeated inoculation on the same culture media. Gram-positive and catalase-negative isolates containing both rod-shaped and coccoid cells were selected.

As a result of the primary screening of rod-shaped LAB isolates, 42 of them with cell morphology, typical to *Lactobacillus delbrueckii* were selected, which grew at different NaCl concentrations (2% and 4%) and at 45°C, but did not grow at 10°C. Meanwhile, gram-positive, catalase-negative cocci in pairs and chains were screened for the growth at different NaCl concentrations (2%, 4% and 6.5%) and temperatures (10°C and 50°C) for the selection of *S. thermophilus* strains. Out of 32 isolates tested, 5 did not grow at 4% NaCl and 10°C, as it is characteristic of this species [9].

For the molecular identification of the selected isolates, DNA was extracted by boiling method [10], with a slight modification, which implies the use of TE buffer (Invitrogen) instead of water and the fermentation temperature of 100°C instead of 95°C. For the identification of *L. delbrueckii*, a pair of primers LB1F: 5' AAAAATGAAGTTGTTTAAAGTAGGTA-3'; LB1R: 5'-AAGTCGTCCTCTGGCTGG-3', and for the identification of *Streptococcus thermophilus,* a pair of primers Th I (5'-ACGGAATGTACTTGAGTTTC-3') and Th II (5'-TTTGGCCTTTCGACCTAAC-3') were used. Polymerase chain reaction (PCR) were performed in a total volume of 25 µl, containing 12.5 µl 2×PCR BIO Tag Mix (PCRBIOSYSTEMS), LB1F/ LB1R: or Th I /ThII at final concentration 0.4 µM each and 2 µl of DNA-containing supernatant. Cycling conditions for LB1F/ LB1R primers: 94°C 2 min, followed by 35 cycles of 94°C for 45 s, 58°C for 30 s and 72°C for 30s, with a final elongation step at 72°C for 10 min, cooling 4°C.

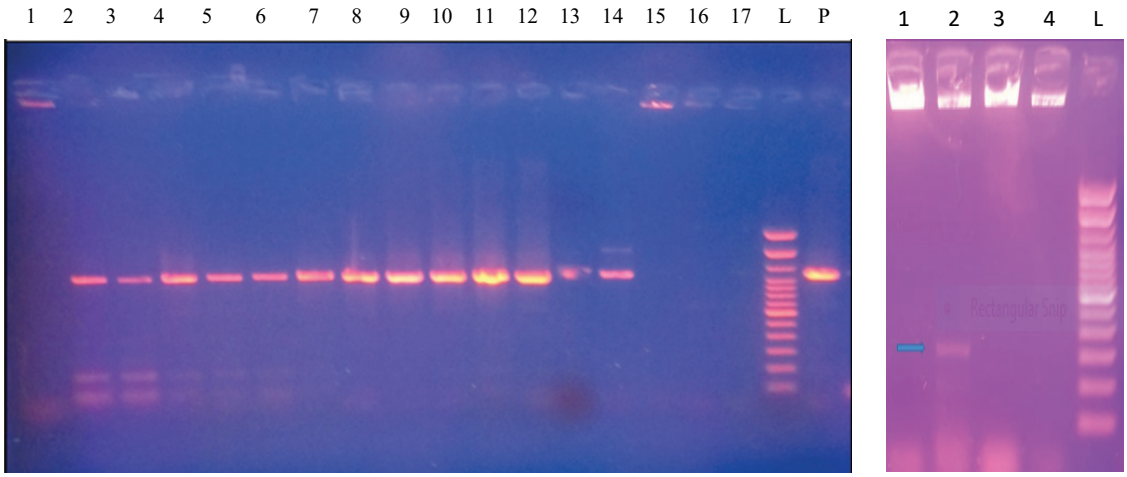

Figure. Electrophoregram of PCR-fragments of some studied LAB isolates: Left - amplified with a pair of *Lactobacillus* specific primers (LB1F/ LB1R), isolate ##: 1 – 51; 2 – T-190; 3 – 191; 4 – 192; 5 – 194; 6 – 198; 7 – 200; 8 – 201; 9 – 205; 10 – 207; 11 – 210; 12 – 211; 13 – 215; 14 –T-221; 15 – 222; 16 – 223; 17 – 224; L – ladder (1000 bp), P – positive control; Right – amplified with a pair of *Streptococcus thermophilus* specific primers (Th I and Th II), isolates ##: 1 – 89; 2 – T-365; 3 – 106; 4 – 111; L – ladder.

Cycling conditions for Th I /ThII primers: 94°C 3 min, followed by 30 cycles of 94°C for 30 s, 50°C for 1min and 72°C for 1 min, with a final elongation step at 72°C for 7 min, cooling 4°C. PCRs were conducted in the thermocycler (TECHNE, TC 3000, US). PCR fragments with primers were visualized on 1.5% agarose gel by horizontal electrophoresis.

Specific primers LB1F/LB1R differentiate two subspecies of *L. delbrueckii*: *L. delbrueckii* ssp. *bulgaricus* and *L. delbrueckii* ssp. *Lactis,* based on the size of PCR: amplified fragment in the 1065 bp and 1600 bp, respectively [11]. Out of the 42 isolates studied, 19 were identified as *L. delbrueckii* ssp. *bulgaricus,* and 2 – as *L. delbrueckii* ssp. *lactis* (Figure-Left). Based on the PCR amplicon size 259 bp, 5 out of 32 studied isolates with coccoid cells were identified as *S. thermophilus* (Figure-Right).

To assess the viability of the selected isolates at low pH their 36 h inoculums were incubated at pH 2 for 30 min, 1 h and 2 h; 100 µl of such inoculums were plated on MRS agar [12]; The observed growth of LAB isolates suggests their tolerance to low pH. After 48 hours of incubation at 37°C under anaerobic conditions, 11 *Lactobacillus* and 3 *Streptococcus* strains retained their viability. These strains also revealed tolerance to 1% bile salts concentration. The proteolytic activity (the ability to form bright areas on non-fat milk agar) in more or less intensity was characteristic to all identified strains.

To study the acidification ability of each selected isolate, their suspensions (10^8-10^9 CFU/ml) were added to non-fat reconstituted, homogenized, pasteurized milk. Fermentation was carried out in 50 ml glass jars with lids at 42°C. Different isolates required different times for thickening/coagulation of milk, which then were stored at 4°C. Variants, with a milk coagulation time of 5-6 hours, were selected for tasting. After the ending of fermentation, the titratable acidity (˚T) of the samples was 98-100. After 8-hour delay in the refrigerator for the fermented milk products, the titratable acidity, pH and organoleptic parameters (the texture, aroma and taste) were evaluated (Table).

The antimicrobial activity of 4 strains, the fermented products of which were considered the best in terms of texture, thickness, taste and aroma, was studied. These strains are: *S. thermophilus* T-365, *S. thermophilus* G-26, *L. delbrueckii* subsp. *bulgaricus* T-190 and *L. delbrueckii* subsp. *lactis* T-221. The selected strains exhibited antimicrobial

Table. Selected LAB strains and characteristics of fermented milk product

Strain #	Origin	Fermented product		
		Titratable acidity, °T	pH	Organoleptic properties
T-190	Kartli region,vicinity of Tbilisi, village Dighomi, bovine milk	130	3.80	Thickness – good, whey – little, taste – mild, pleasant
T-365	Imereti region, Khoni munitsipality, village Gordi, whey remaining after cheese-making	150	3.81	Thickness – good, taste and smell – pleasant
G-26	Samtskhe-Javakheti region, Aspindza munitsipality, village Chobareti, traditional Georgian cheese Tenili ripened in a clay pot	140	3.86	Thickness – good, taste and smell – pleasant
T-221	Kartli region, vicinity of Tbilisi, village Oqrokhana, bovine milk	130	3.84	Texture – good, taste and smell- pleasant
327	Tbilisi, aboveground parts of plant Achillea	150	3.81	Texture – good, taste – bitter
203	Samtskhe-Javakheti region, Borjomi munitsipality, village Bakuriani, bovine milk	150	4.10	Thickness – good, taste-sour-bitter

activity of varying intensity to almost all tested-cultures.

Thus, as a result of multi-step screenings according to the probiotic, biotechnological and organoleptic characteristics, following 4 autochthonous Georgian LAB strains were selected: *S. Thermophilus* T-365, *S. thermophilus* G-26, *L. delbrueckii* ssp. *bulgaricus* T-190 and *L. delbrueckii* ssp. *Lactis* T-221. These strains can be used, either individually or in combination, for a milk fermentation to obtain Georgian topical fermented milk beverages.

ბიოტექნოლოგია

საქართველოს სხვადასხვა რეგიონში გამოყოფილი რძემჟავა ბაქტერიების პრობიოტიკული თვისებები და ბიოტექნოლოგიური პოტენციალი

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ბოლო დროს პრობიოტიკების სამყაროს სწრაფი ექსპანსიის მიუხედავად, აქტიურად მიმდინარეობს ახალი სტარტერი კულტურების ძიება რძემჟავა ბაქტერიებს შორის. სამუშაოს მიზანს წარმოადგენდა საქართველოს ავტოქტონური რძემჟავა ბაქტერიების გამოყოფა და მათი ზოგიერთი პრობიოტიკული თვისებისა და ბიოტექნოლოგიური პოტენციალის შესწავლა შემდგომში მათ საფუძველზე რძის ფერმენტირებილი სასმელი პროდუქტის შესაქმნელად. ჩვენ მიერ რძემჟავა ბაქტერიების გამოყოფა განხორციელდა საქართველოს სხვადასხვა რეგიონში შეგროვებული ნედლი, თვითჩადედებული რძიდან, ყველის შრატისა და მცენარეების ფილოსფეროდან; სულ 116 ნიმუშიდან გამოყოფილ იქნა 263 იზოლატი. მორფოლოგიური, ფიზიოლოგიური და ბიოქიმიური ტესტების მიხედვით შერჩეული იზოლატების 16S რდნმ-ების პჯრ-პროდუქტების შესწავლის შედეგად, მათ შორის გამოვლინდა *L. Delbrueckii*-ის 21 და *S. thermophilus*-ის 5 შტამი. მრავალსაფეხურიანი სკრინინგის შედეგად, პრობიოტიკული, ბიოტექნოლოგიური და ორგანოლეპტიკური მაჩვენებლების შესწავლის საფუძველზე შეირჩა 4 ავტოქტონური შტამი: *S. thermophilus* T-365, *S. thermophilus* G-26, *L. delbrueckii* subsp. *bulgaricus* T-190 და *L. delbrueckii* subsp. *lactis* T-221, რომლებიც ცალკე ან გარკვეული კომბინაციებით შესაძლოა გამოყენებულ იქნეს ადგილობრივი რძის ფერმენტირებული სასმელი პროდუქტის მისაღებად.

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