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Қ. И. Сәтпаев атындағы Қазақ ұлттық техникалық зерттеу университеті

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ИЗВЕСТИЯ

НАЦИОНАЛЬНОЙ АКАДЕМИИ НАУК
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Қазақстан Республикасы Ұлттық ғылым академиясы "ҚР ҰҒА Хабарлары. Геология және техникалық ғылымдар сериясы" ғылыми журналының Web of Science-тің жаңаланған нұсқасы Emerging Sources Citation Index-те индекстелуге қабылданғанын хабарлайды. Бұл индекстелу барысында Clarivate Analytics компаниясы журналды одан әрі the Science Citation Index Expanded, the Social Sciences Citation Index және the Arts & Humanities Citation Index-ке қабылдау мәселесін қарастыруда. Webof Science зерттеушілер, авторлар, баспашылар мен мекемелерге контент тереңдігі мен сапасын ұсынады. ҚР ҰҒА Хабарлары. Геология және техникалық ғылымдар сериясы Emerging Sources Citation Index-ке енуі біздің қоғамдастық үшін ең өзекті және беделді геология және техникалық ғылымдар бойынша контентке адалдығымызды білдіреді.

НАН РК сообщает, что научный журнал «Известия НАН РК. Серия геологии и технических наук» был принят для индексирования в Emerging Sources Citation Index, обновленной версии Web of Science. Содержание в этом индексировании находится в стадии рассмотрения компанией Clarivate Analytics для дальнейшего принятия журнала в the Science Citation Index Expanded, the Social Sciences Citation Index и the Arts & Humanities Citation Index. Web of Science предлагает качество и глубину контента для исследователей, авторов, издателей и учреждений. Включение Известия НАН РК. Серия геологии и технических наук в Emerging Sources Citation Index демонстрирует нашу приверженность к наиболее актуальному и влиятельному контенту по геологии и техническим наукам для нашего сообщества.

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IDENTIFICATION OF PROBIOTIC STRAINS BY MODERN ANALYTICAL TECHNIQUES

Abstract. A total of 14 dominant lactic acid bacteria (LAB) isolates were recovered from traditional milk products, such as camel milk, shubat, koumiss and kurt produced in the Republic of Kazakhstan in an effort to screen novel potential starter strains. In order to identify the isolates at the species level API 50 CH carbohydrate fermentation tests, MALDI TOF mass-spectrometry and 16S ribosomal RNA (rRNA) sequence analysis were performed. Four pure *Bifidobacterium* strains were isolated from a camel milk and they were identified as *Bifidobacterium crudilactis* using the 16S rRNA gene sequencing. On the other site, our results demonstrated firstly the presence of the genus *Bifidobacterium* in camel milk.

Keywords: probiotic, camel milk, shubat, koumiss, kurt, fructose-6-phosphate phosphoketolase (F6PPK), 16S rRNA sequencing.

Introduction. Lactic acid bacteria (LAB) have a long history of safe use in food fermentations and play a significant role in the manufacture of fermented milk products [1, 10]. Dairy industry is constantly exploring new possibilities to increase the diversity of dairy products in order to meet the technological needs and consumer demands. Therefore, there is a growing interest in searching for potential novel starter microorganisms from various unconventional sources, such as raw milk products [16,20] and many traditional dairy foods such as fermented yak milk and goat milk, koumiss, butter, cheese, kefir, whey and qula [2, 4, 7, 9, 11, 14, 15, 19, 21, 22]. In this paper, we describe the lactic acid bacteria isolated from three traditional Central Asian dairy products, koumiss, shubat and kurt, each manufactured by a simple backslopping procedure.

Koumiss is a fermented dairy product traditionally made from mare's milk. The drink remains important to the peoples of the Central Asian steppes, of Huno-Bulgar, Turkic and Mongol origin: Kazakhs, Bashkirs, Kalmyks, Kyrgyz, Mongols and Yakuts.

The artisanal manufacture of koumiss is based on the inoculation of raw mare's milk with previously produced koumiss. Today koumiss is made from pasteurized mare's milk by adding about 30% the previous batch, and allowing to ferment at 26-28 °C for 2-4 hours with occasional stirring. After fermentation, koumiss is bottled, capped, and stored for ripening at 4-6°C for 1-3 days. The major fermentation products are lactic acid, ethanol and carbon dioxide.

Shubat is a fermented camel milk beverage and a favorite part of daily diet for many people of Kazakhstan. Traditionally, shubat is considered useful against such diseases as asthma, tuberculosis, hepatitis, diabetes, and psoriasis. The process of shubat preparation is simple and - like tahat of koumiss - is based on backslopping. Fresh camel milk is poured into wooden container, previously made shubat is added, the container is covered and the milk left to ferment.

Kurt is a Kazakh national product manufactured from pasteurized cow, sheep or goat milk. The product is made from low-fat milk, which is pasteurized at 80-85 °C (176-185 °F) for 10-20 sec, then cooled to 32-34 °C (89.6-93.2 °F) and inoculated with the starter culture. The starter culture consisting of

Lactococci, *Streptoccocus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* is added at a rate of 5%. The milk is incubated until the acidity reaches 75-76°Th (0.67-0.68 % titratable acidity).

Then the coagulum is heated to 38-42 °C (100.4- 107.6°F) and held at this temperature for 20-30 min to facilitate whey separation. After whey drainage the kurt is put in cloth bags, 7-9 kg at a time, and pressed until the moisture content reaches 76-80%, usually within 3-5 h. After pressing, the kurt is molded into pieces of irregular shapes weighing 25-60 g, with or without prior salting, and then air-dried at 35-40°C (95-104°F) until the moisture content is 17% or less. The final product contains not more than 17% moisture, 12% fat in the dry matter, and a maximum of 2,4% salt. It may be reconstituted with water into a beverage [3].

Here we report the taxonomic characterization of some dominant LAB species recovered from camel milk, shubat, koumiss and kurt in an effort to screen for novel potential starter strains adapted to grow in these types of products.

The methods of researches.

Sample collection. The camel raw milk, shubat and koumiss samples were collected from small villages located near the city of Almaty, Kazakhstan during the period in summer 2016. Artisanally produced kurt was collected from a farm in Kyzylorda region (Kazakhstan). After measuring the pH of the products, samples were collected aseptically into sterile tubes, kept in an ice-box and transported for analysis to the microbiology laboratory of Czech University of Life Sciences, Prague (table 1).

Table 1 – Lactic acid bacteria strains, sourse of isolation and origin

Sample	Sourse of isolation	Origin
5-2M	Shubat	Kazakhstan, Almaty
5-5M		
6-2M	Koumiss	Kazakhstan, Almaty
6-12M		
7-1M	Camel milk	Kazakhstan, Almaty
7-4M		
7-8M		
7-1C		
7-2C		
7-5C		
7-6C		
8-2M	Kurd	Kazakhstan, Kyzylorda
8-6M		
8-9M		

Isolation and cultivation of strains. One ml or 1 g of each product was mixed with 9 ml of 0.85% (w/v) sterile physiological saline. Serial dilutions were made for each sample plated in triplicate on universal and selective media: MRS agar (Difco™) for Lactobacilli and for Bifidobacteria Wilkins-Chalgren agar (Oxoid, UK) supplemented with the soybean peptone (5g/L, Oxoid), L-cysteine-HCl (0.5 g/L, Sigma-Aldrich), Tween 80 (1 mL/L, Sigma-Aldrich). Selective agents mupirocine (100 mg/L, Oxoid) and glacial acetic acid (1 mL/L; Sigma-Aldrich) were also included this medium (Rada and Petr, 2000; Ferraris et al., 2010). The plates were incubated anaerobically (BBL GasPak 100 Anerobic system, BD Biosciences, Sparks, MD, USA) at 37°C for 2–3 days. Representative single colonies from the highest dilutions were randomly selected from the agar plates and transferred into tubes containing MRS broth and anaerobic Wilkins-Chalgren broth. The isolates were cultivated for 24 h at 37 °C. Purity was checked using the light microscope.

Phenotypic studies. The biochemical properties of the strains were characterized using API 50 CHL strips (Biomerueux, France) according to the instructions provided by the manufacturer. For suspected bifidobacteria, the phosphoketolase assay was used as a diagnostic test. The cells were washed twice

(10 000×*g*, 4°C, 15 min) with phosphate buffer [0.05 M KH_2PO_4, mixed 1:1 with cysteine·HCl, (500 mg/liter), pH adjusted to 6.5 with fresh NaOH] and resuspended in 1.0 ml of phosphate buffer. Washed bacterial cells either underwent no pretreatment, sonication for 6 min in ice, or were incubated with Cetyl Trimethylammonium Bromide (CTAB) for 5 min prior to the assay. CTAB was added in graded amounts of 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6 and 0.7 ml (450 μg/ml stock solution) to determine the level of CTAB that would be effective for cell disruption.

After pretreatment, 0.25 ml of a solution containing sodium fluoride (NaF, 3 mg/ml) and potassium or sodium iodoacetate (5 mg/ml) in H_2O was added, followed by 0.25 ml sodium fructose-6-phosphate solution (80 mg/ml in H_2O). The mixture was vortexed and then incubated at 37°C for 30 min. After incubation, 1.5 ml of hydroxylamine·HCl (13 g/100 ml) was added, and the vortexed mixture was incubated at room temperature for 10 min. One milliliter of TCA (15%, W/V), 1.0 ml of 4N HCl and 1.0 ml of ferric chloride ($FeCl·6H_2O$, 5% W/V in 0.1 N HCl) were added, the tubes were vortexed and color formation was recorded either using a qualitative scale or spectophotometrically at 505 nm. For spectrophotometric determinations, the stopped reaction mixture was centrifuged (10 000×*g*, 4°C, 15 min) and the supernatant was measured using a Bausch and Lomb Spectronic 70 spectrophotometer. Test tubes containing reagents without cells, or cell suspensions with all the reagents except fructose-6-phosphate, were used as blanks.

Mass-spectrometric analysis was carried out using Autoflex MALDI TOF mass-spectrometer (Bruker Daltonics, Germany). Fresh overnight cultures and ethanol–formic acid extraction procedure were applied for the microorganism profiling. Each sample spot was overlaid with 2 μL of matrix solution (saturated solution of α-cyano-4-hydroxy-cinnamic acid in 50% acetonitrile with 2.5% trifluoroacetic acid) and again air-dried for 15 min (Bruker Daltonik GmbH, Germany). To identify the microorganisms, the raw spectra obtained for each isolate were imported into the BioTyper software, version 2.0 (Bruker Daltonik) and analyzed without any user intervention.

Identification based on 16S rRNA gene sequencing. Genomic DNAs from the bacterial strains were obtained using the commercial Genomic DNA Mini Kit applied biosystem *PrepMan Ultra* Sample Preparation Reagent (Applied Biosystems). Almost complete 16S rRNA gene fragment was amplified using primers 616V and 630R under PCR conditions as described previously [12]. Amplifications were performed with a thermal cycler (Biometra T professional gradient Thermocycler) and then confirmed using the 1.5% agarose electrophoresis (110V, 30 minutes) under the UV lamp. Checked samples were purified using the PCR purification kit (Qiagen) and sent to the GATC (Biotech company) for sequencing. The complete 16S rRNA gene sequences were reconstructed on the basis of 2 sequences, derived from forward and reverse primer. For these purposes, the BioEdit v7.2.5 program (available at: http://www.mbio.ncsu.edu/bioedit/page2.html) was used. Identification of all 14 strains was performed by comparison of obtained sequences with all 16S rRNA gene sequences included in EzTaxon database [8]. Assembly of genome and bioinformation analysis was carried out using BLAST program (www.blast.ncbi.nlm.nih.gov/blast.cgi). Obtained sequences were deposited in GenBank database using the BanKlt system (https://www.ncbi.nlm.nih.gov/WebSub/?tool=genbank) [13].

Results. Altogether 14 isolates representing the dominant colony types in the different milk and dairy product samples recovered from different geographical regions were selected for further study. The isolate codes, their species designations suggested by the different identification methods are listed in Table 2.

It can be seen that members of *Lactobacillus* and *Lactococcus* genera dominated the dairy food samples, while *Bifidobacterium* was a common finding in the raw camel milk (present at levels of 10^6 CFU per ml). Although bifidobacteria are often detected in raw milk [6], this finding was somewhat surprising, because no selective enrichment was applied before the plating in our study.

In order to confirm the species designation, the presumed *Bifidobacterium* isolates were tested for fructose-6-phosphate phosphoketolase activity. The fructose-6-phosphate phosphoketolase or transketolase assay is considered as a definitive biochemical test for the *Bifidobacterium* genus [17,18]. A limitation of the assay is the time-consuming process of cell disruption, either by use of the French Pressure Cell or by sonication. Accordingly, we replaced the cell disruption process with a more rapid cell membrane disruption process by pretreating cells with the detergent hexadecyltrimethylammonium bromide (cetrimonium bromide, CTAB). According to our results (unpublished data), the CTAB treatment produced results identical to those obtained with the conventional cell disruption procedure with known positive (*Bifidobac-*

terium) and negative (*Lactobacillus*) controls. The positive reaction (formation of reddish-violet color) seen with the isolates 7-1C, 7-2C, 7-5C and 7-6C confirmed the identification as *Bifidobacterium* as indicated by the 16S rRNA sequence data suggesting *B. crudilactis* as the species. This species has been previously identified as a novel species isolated from French raw milk and raw milk cheeses [5].

Table 2 – Phenotypic and genetic characterization of lactic acid bacteria

Isolate	Appr. CFU count per g or ml	Suggested species identity		
		API 50 CHL	MALDI- TOF	16s rRNA
5-2M	$6{,}6\times10^{7}$	*Lactobacillus helveticus*	*Lactobacillus ultunensis*	*Lactobacillus helveticus*
5-5M	$5{,}6\times10^{8}$	*Lactobacillus brevis*	not reliable identification	*Lb. pontis*
6-2M	$1{,}0\times10^{7}$	*Lactobacillus acidophilus*	*Lactobacillus kefiranofaciens*	*Lb kefiranofaciens*
6-12M	$1{,}6\times10^{7}$	*Lb. pontis*	not reliable identification	*Lb. pontis*
7-1M	$1{,}2\times10^{8}$	*Lact.lactis* subsp. *lactis*	*Lactococcus lactis*	*Lactococcus lactis* subsp. *lactis*
7-4M	$6{,}5\times10^{7}$	*Lact.lactis* subsp. *lactis*	*Lactococcus lactis*	*Lact.lactis* subsp. *hordiane*
7-8M	$2{,}9\times10^{8}$	*Lact.lactis* subsp. *lactis*	*Lactococcus lactis*	*Lact.lactis* subsp. *lactis*
7-1C	$2{,}6\times10^{7}$	*Lactobacillus brevis*	not reliable identification	*Bifidobacterium crudilactis**
7-2C	$4{,}8\times10^{6}$	*Lactobacillus brevis*	not reliable identification	*Bif.crudilactis**
7-5C	$2{,}2\times10^{8}$	*Lactobacillus brevis*	not reliable identification	*Bif.crudilactis**
7-6C	$1{,}7\times10^{8}$	*Lactobacillus brevis*	not reliable identification	*Bif.crudilactis**
8-2M	$9{,}2\times10^{7}$	*Lactobacillus paracasei*	*Lactobacillus paracasei*	*Lb. paracasei*
8-6M	$2{,}0\times10^{7}$	*Lactobacillus brevis*	*Lactobacillus brevis*	*Lb. brevis*
8-9M	$5{,}6\times10^{7}$	*Lactobacillus brevis*	*Lactobacillus paralimentarius*	*Lb. crustorum*
*Confirmed by phosphoketolase assay.				

Discussion. The traditional fermented milk products from Central Asia have a reputation of being both highly nutritious and also health promoting. However, the evidence has largely been anecdotical, and serious investigations of the role of lactic acid bacteria or their metabolic products in the presumed health effects have only recently been started.

The technological suitability and actual probiotic efficacy of these strains have to be thoroughly assessed before their eventual applications, our results confirm the great potential of traditional Kazakh milk products as a source of novel dairy starters or probiotic cultures, as indicated in some recent studies on similar products from other geographical regions.

Conclusion. The present study represents the microbiological investigation of the traditional milk products, such as camel milk, shubat, koumiss and kurt produced in the Republic of Kazakhstan. Our results indicate the occurrence of the species *B. crudilactis* in camel milk originated from Kazakhstan area. A total of 14 dominant lactic acid bacteria (LAB) isolates were assessed for their technical suitability as starters and particularly their probiotic properties.

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ПРОБИОТИКАЛЫҚ ШТАМДАРДЫ ЗАМАНАУИ АНАЛИТИКАЛЫҚ ӘДІСТЕРМЕН ИДЕНТИФИКАЦИЯЛАУ

Аннотация. Қазақстан Республикасының түйе сүті, шұбат, қымыз және құрт тәрізді дәстүрлі сүтқышқылды өнімдерінен белсенді жаңа штамдарды скринингілеу мақсатында жалпы саны 14 доминанты өсінділер бөлініп алынды. Өсінділерді түрге дейін ажырату үшін көмірсулардың ферменттелу API 50 CH тесті, MALDI TOF масс-спектрометриясы және 16S рибосомалық РНҚ (рРНҚ) дәйектілігі бойынша талдау жүргізілді. Bifidobacterium тобының 4 жаңа штамдары түйе сүтінен бөлініп алынып, олар 16S рРНҚ генін секвенирлеу арқылы Bifidobacterium crudilactis екені анықталды. Екінші жағынан, біздің зерттеу нәтижелеріміз алғашқы рет түйе сүтінде Bifidobacterium тобының кездесетініндігін дәріптейді.

Түйін сөздер: пробиотик, түйе сүті, шұбат, қымыз, құрт, фруктоза-6-фосфат-фосфокетолаза (F6PPK), 16S р РНҚ секвенирлеу.

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ИДЕНТИФИКАЦИЯ ПРОБИОТИЧЕСКИХ ШТАММОВ СОВРЕМЕННЫМИ АНАЛИТИЧЕСКИМИ МЕТОДАМИ

Аннотация. В общей сложности 14 доминантных изолятов молочнокислых бактерий были выделены из традиционных молочных продуктов, таких как верблюжье молоко, шубат, кумыс и курт, произведенных в Республике Казахстан, в целях скрининга новых потенциальных штаммов заквасок. Для идентификации изолятов на уровне видов были проведены тесты ферментации углеводов API 50 CH, масс-спектрометрия MALDI TOF и анализ последовательности 16S рибосомальной РНК (рРНК). Четыре чистых штамма Bifidobacterium были выделены из верблюжьего молока, и они были идентифицированы как Bifidobacterium crudilactis с использованием секвенирования гена 16S рРНК. С другой стороны, наши результаты первый раз продемонстрирует наличие рода Bifidobacterium в верблюжьем молоке.

Ключевые слова: пробиотик, верблюжье молоко, шубат, кумыс, курт, фруктоза-6-фосфат-фосфокетолаза (F6PPK), 16S р РНК секвенирование.

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