Islamic Azad University
Urmia Branch

Journal of Basic and Clinical Veterinary Medicine

2023; 4(1): 12-18

Official Journal of Veterinary Faculty of Islamic Azad University
Urmia Branch

Journal Homepage: jbcvm.iaurmia.ac.ir

Original Article

Isolation and identification of *Lactobacillus* species from donkey milk in the Azerbaijan region of Iran using 16S rDNA gene sequencing

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ARTICLE INFO

Received: 15 February 2023

Accepted: 18 March 2023

DOI:10.30495/jbcvm.2023.1980303.1040

KEYWORDS:

Donkey milk
Lactobacillus species
16S rDNA sequencing
Azarbaijan region of Iran

ABSTRACT

The use of donkey milk is increasing due to its nutritional properties and lack of allergenic proteins. The present research was conducted with the aim of identifying native *Lactobacillus* bacteria. Three samples of donkey milk were collected from Benab, Maragheh, and Naqhadeh cities of the Azerbaijan region randomly and in heed with sterile conditions. The samples were cultured in MRS agar and specific tests were performed on the grown colonies to identify *Lactobacillus*. Molecular identification of the isolates was done based on the amplification of the 16S rDNA gene using specific primers and polymerase chain reaction. *Lactobacillus* species were analyzed by sequencing the 16S rDNA gene and drawing a phylogenic tree. Based on PCR results, 3 isolates of *Lactobacillus* were detected. The results of sequence analysis showed that two isolates are highly similar to *Lactobacillus plantarum* and one isolate to *Lactobacillus fermentum*. Therefore, it can be concluded that *Lactobacillus plantarum* is the dominant species in donkey milk in the Azerbaijan region of Iran. Due to the probiotic potential of *lactobacillus* isolates from donkey milk, it is suggested to be used in the production of probiotic milk products.

جداسازی و شناسایی گونه‌های *لاکتوباسیلوس* از شیر الاغ منطقه آذربایجان ایران با استفاده از تعیین توالی ژن 16S rDNA

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چکیده

استفاده از شیر الاغ به دلیل خواص تغذیه‌ای و فقدان پروتئین‌های آلرژی‌زا در حال افزایش است. تحقیق حاضر با هدف شناسایی باکتری‌های *لاکتوباسیلوس* بومی انجام شد. تعداد ۳ نمونه شیر الاغ از شهرهای بناب، مراغه و نقده منطقه آذربایجان به صورت تصادفی و با رعایت شرایط سترون جمع‌آوری شدند. نمونه‌ها در آگار MRS کشت و بر روی پرگنه‌های رشد یافته، آزمایش‌های اختصاصی جهت شناسایی لاکتوباسیلوس انجام شد. شناسایی مولکولی جدایه‌ها، براساس تکثیر ژن ۱۶S rDNA با استفاده از پرایمرهای اختصاصی و واکنش زنجیره‌ای پلیمراز انجام شد. گونه‌های *لاکتوباسیوس* با تعیین توالی ژن ۱۶S rDNA و ترسیم درخت فیلوژنی آنالیز شدند. براساس نتایج PCR، ۳ جدایه *لاکتوباسیلوس* تشخیص داده شدند. نتایج آنالیز توالی نشان داد که دو جدایه با *لاکتوباسیلوس پلانتاروم* و یک جدایه با *لاکتوباسیلوس فرمنتوم* مشابهت بالایی دارند. بنابراین می‌توان نتیجه‌گیری نمود که *لاکتوباسیلوس پلانتاروم* گونه غالب در شیر الاغ منطقه آذربایجان ایران می‌باشد. به دلیل پتانسیل پروبیوتیکی جدایه‌های *لاکتوباسیلوس* شیر الاغ پیشنهاد می‌گردد در تولید فرآورده‌های شیر پروبیوتیکی استفاده گردند.

واژه های کلیدی: شیر الاغ، گونه‌های لاکتوباسیلوس، تعیین توالی ۱۶S rDNA، منطقه آذربایجان ایران

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INTRODUCTION

Recently, donkey milk has attracted scientific attention because of its nutrients and physiological properties, including immunoglobulins and other immune system proteins, digestive system by enzymes and enzyme inhibitors, production of growth factors, and microbiological characteristics such as low microbial load and minor pathogens [1-3]. In addition, in recent years, researchers have shown that donkey milk components have anticarcinogenic, antiviral and antibacterial effects [4]. Donkey milk has low protein and casein, little fat content, and high levels of lactose [5]. Human milk and donkey milk share close similarities in terms of the levels of lactose value. [6]. Hence, if the mother cannot breastfeed her infant, cow's milk is one of the best and first solutions for parents [7]. Intolerance to cow's milk and sensitivity to it may be mild, moderate to severe. [8]. These complications have prompted nutritionists to study the substitution of donkey milk with cow milk [9].

Probiotics are beneficial bacteria that can occupy different habitats such as gut, oral cavity, breast, skin, etc. in humans [10, 11]. The probiotics enhance intestinal digestion and absorption function of certain nutrients, and can destroy various pathogens [12, 13]. Lactobacillus acidophilus is the best-known probiotic and one of the most important probiotics for health [14]. L. acidophilus inhibits pathogens and produces potency natural antibiotics such as lactocidin and acidophilin, which increase immunity [15]. In addition, the antimicrobial effects of L. acidophilus have been reported against salmonella, staphylococcus aureus, E. coli and candida albicans [16].

Isolation, identification, and screening of microorganisms from natural sources are effective means of obtaining genetically important species of bacteria [17]. Lactic acid bacteria (LAB) play an effective role in improving human living conditions [18]. Dairy products are the leading source of these bacteria [18]. Considering the importance of lactic acid-producing bacteria in health and their use in the food industry, it is very important to search for novel species from different sources. There is little published research on donkey milk microflora. The present study was conducted to isolate and identify Lactobacillus species in the Azerbaijan region of Iran using the 16S rDNA sequencing technique.

MATERIALS AND METHODS

Sampling

Three donkey milk samples were randomly collected from Bonab, Maragheh, and Naghadeh cities in the Azerbaijan region of Iran with sterile conditions in 2022. The samples were stored in the laboratory at 4 °C and were cultured bacteriologically as soon as possible.

Isolation and preliminary identification of Lactobacilli

For Lactobacilli isolation, the pH of the samples was first measured, and the samples were serially diluted in a sterile saline solution. Then, 0.1 ml of the diluted samples were spread on de Man, Rogosa, and Sharpe (MRS) agar (Quelab company, Montreal, Canada). Inoculated agars were incubated at 37°C for 48-72 h under anaerobic conditions. Then,

Gram staining and catalase test were performed on the isolated colonies. The colonies with rod-shaped gram-positive and catalase-negative characteristics were selected and sub-cultured on MRS agar. The purified presumptive Lactobacillus colonies were inoculated in MRS broth and incubated in the same conditions. Then, after adding 15% glycerol, the stock cultures were stored at -80°C.

DNA extraction of the presumptive Lactobacillus isolates

To extract the genomic DNA, presumptive Lactobacillus isolates were cultured in MRS broth and incubated at 37°C for 48-72 h in anaerobic conditions. After the incubation, the culture was centrifuged at 10000 rpm for 5 min and, the supernatant was discarded and washed twice with double-distilled water. The genomic DNA of Lactobacilli species was isolates using the published protocol of the SinaPure DNA kit (CinnaGen company, Tehran, Iran). The universal primers were used for the amplification of the 16S rDNA gene of the isolates by PCR as follows: 5´-AGCAGTAGGGAATCTTCCA-3´ (forward) and 5´- ATTYCACCGCTACACATG-3´ (reverse). The PCR amplification was performed using a thermal cycler (Analytik Jena AG, Germany) with the 25 µl final volume of the reaction mixture, including 12.5 µl master mix (CinnaGene, Tehran, Iran), 50 ng/ml chromosomal DNA, and 5pmol of each primer. The PCR thermal cycler was programmed with the initial conditions contained denaturation at 95 ˚C for 5 minutes followed by 35 cycles of denaturation at 95 ˚C for 60 seconds, primer annealing at 59 ˚C for 60 seconds and primer extension at 72 ˚C for 2 minutes, and a final extension at 72 ˚C for 5 minutes. The PCR products were separated by electrophoresis on 1.0% (w/v) agarose gel containing red safe (concentration from 0.8-3.0%), and DNA ladder marker with 100bp (CinnaGene, Tehran, Iran) for one hour in 1× tris-acetate-EDTA (TAE) buffer at the constant voltage of 85 V. In addition, the PCR product was visualized with the ultraviolet fluorescence gel documentation system (UVITEC, England, United Kingdom) [17].

Sequencing and phylogenetic analysis

The PCR products were sequenced by the Takapouzist Co (Tehran, Iran). Moreover, the sequences analysis of the 16S rDNA gene of Lactobacillus isolates, using the NCBI BLAST (http://blast.ncbi.nlm.nih.gov), was compared with the reference of bacterial species included in the GenBank database. Molecular phylogenetic analysis was performed by the maximum likelihood method based on the Tamura-Nei model. All the analyses were conducted on a bootstrap dataset containing 1000 replicates. Evolutionary analyses were performed in MEGA X software [17].

RESULTS

The pH value of the donkey's milk for Naqadeh, Bonab, and Maragheh was 7.1, 7.3, and 7.2, respectively. Among the presumptive Lactobacillus isolates, the PCR results indicated that three isolates had a 345 bp specific band in electrophoresis (Figure 1). The alignment of the nucleotide sequencing (345 bp) of three isolates (P2, P10, and P11) in the gene bank using NCBI and the relationship between strains of Lactobacillus species were evaluated by MEGA X software to compare the DNA sequencing generated by each primer represented that P2 and P10 isolates shared high similarity with Lactobacillus plantarum. P11 isolate belonged to Lactobacillus fermentum (Figure 2).

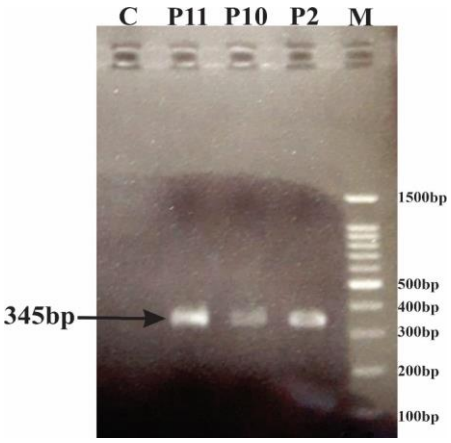

Figure 1. The *apoE* gene expression in the PBMC of rats receiving betaine and water .GAPDH was used as an internal control gene. The quantified amount was depicted in charts.

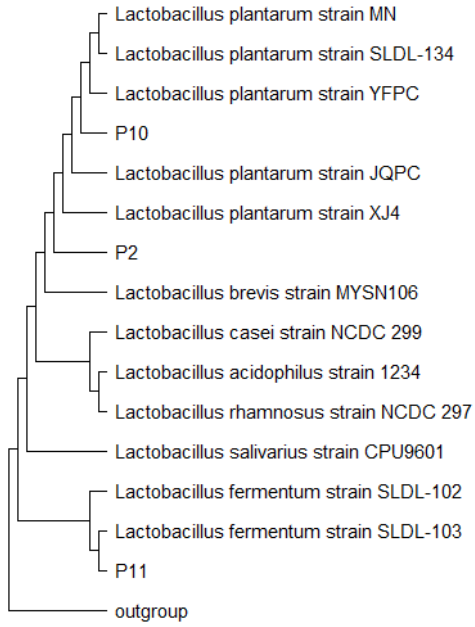

Figure 2. 16S rDNA tree showing the phylogenetic relationship by maximum likelihood method based on the Tamura-Nei model between three isolates with other known Lactobacillus species.

DISCUSSION

In the last few years, donkey milk has been widely studied [19]. Most of the studies were conducted in areas where donkey raising is a tradition [20]. The non-allergic properties of donkey milk combined with the aforementioned reasonshave led to its high consumption among humans worldwide [20]. The LAB includes various groups of microorganisms that are naturally present in many foods, especially fermented, and in the gastrointestinal and urogenital systems of animals . *Lactobacillus plantarum* is one of the identified isolates in this research that can produce bacteriocin [21]. Bacteriocins enter the cell and exert their effects by binding to the cell surface, although they are low molecular weight proteins. There are different ways that bacteriocins kill cells, including making holes in the cell, breaking down DNA, and preventing the cell from making proteins [21]. The use of *Lactobacillus plantarum* as a beneficial probiotic bacterium has been studied extensively in the last 20 years [22]. The *L. plantarum* is often used to improve the flavor, texture and shelf life of fermented

foods. The production of lactic acid and other antimicrobial compounds by the LAB plays an important role in production of safe foods [22]. In addition, studies have shown that treatment of cell lines such as H4, PSIc1, and CLAP with *L. plantarum* did not lead to any harmful effects [23]. In this research, *L. plantarum* ,for the first time, is identified and reported in donkey milk in Iran. *L. plantarum* is a highly flexible and fluent microorganism that can be isolated from various sources and has the largest genome (~3.3 Mb) among the *Lactobacillus* genus [24]. *L. plantarum*, as a probiotic bacterium, can be used in many industrial sections such as food and beverage fermentation. Although donkey milk is rich in lysozyme, *L. plantarum* can survive in the presence of lysozyme. Considering that donkey milk is rich in lysozyme, it is logical that it can be found in the microbiota of donkey milk [25]. This survival activity of the *L. plantarum* is attributed to the O-acetylation of peptidoglycan N-acetyl muramic acid (MurNAc) encoded by the oatA gene [20]. However, there is no information about the resistance of phenotype of *L. plantarum* to the high concentration of lysozyme. Therefore, the application of this bacterium in probiotic pills and probiotic dairy products can be beneficial [20]. In the present research, little diversity was observed in the identified *Lactobacillus* species. This low diversity has been reported by other researchers. Therefore, it seems that by increasing the sample number and performing additional bacteriological tests, it is possible to identify the other species of *Lactobacillus.*

CONCLUSION

In the present research, three isolates were identified as *Lactobacillus*. The PCR product sequencing of the 16S rDNA gene and phylogenetic tree analysis showed that two isolates have high similarity to *L. plantarum*, and one isolate belongs to *L. fermentum*. Therefore, *L. plantarum* was the predominant Lactobacillus spp. in donkey milk in the Azerbaijan region of Iran. Having a detailed and complete information about donkey milk microbiota can be beneficial in producing novel and functional foods with health benefits for humans.

AKNOWLEDGMENTS

The authors are grateful to genetic reference and microbiology laboratories, Islamic Azad University, Urmia, Iran, for providing the necessary laboratory facility for this research study.

ETHICS

Approved.

CONFLICT OF INTEREST

None declared.

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