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Original Article

Isolation and Identification of *Lactic Acid Bacteria* from Iranian Camel Milk

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ABSTRACT

Background: Camel milk is the most important of dairy foods. Its contains amino acids, vitamins, probiotics properties and a potential source for isolation of probiotic *Lactobacillus* strains. This study is aimed to identify and isolate the bacteria special *Lactic Acid Bacteria* in the camel milk based on the molecule methods.

Methods: All samples were collected from camel milk in Semnan province of Iran. Initially, they were cultivated in MRS Agar. Plates were incubated by 37 °C for 48 hours. Bacteria identification was done according to interior transcription of the area *16 SrRNA*. The products of PCR were successfully determined and were analyzed. Finally, a phylogenetic tree was constructed by using Clustal Omega.

Results: The observed bacteria were gram-positive, catalase-negative rods or cocci and vancomycin-resistant. Following that they identified as *E. gallinarum* and *E. casseliflavus* by analytical results ribosomal DNA sequencing with 100% similarity. Also, a phylogenetic tree is proven the species relatedness of the *Enterococcus* spp.

Conclusions: These findings showed that supporting *16S rRNA* sequences is a reasonable technique for identifying *Lactobacillus* strains. Also, isolated bacteria are a strong candidate for using in food and pharmaceutical industry.

Key words: *E.casseliflavus*, *E.gallinarum*, *Enterococcus*, Probiotic, *16s rRNA*

Introduction

The genus *Enterococcus* belongs to *lactic acid* bacteria (*LAB*) group of the phylum Firmicutes. They derived from the former genus *Streptococcus*. *Enterococci* are facultatively anaerobic, Gram-positive cocci, non-spore forming, and catalase-negative (Liu *et al*., 2014).

Lactic acid bacteria are extensively distributed in the soil, plants, insects, birds, humans, animals, water, which they used for the fermentation and preservation of a wide range of milk, meat and vegetable foods (Kayser, 2003). The *LAB* including *Lactococcus*, *Streptococcus*, *Lactobacillus*, *Leuconostoc*, *Pedicoccus* and several *Enterococcus* (Saranraj

et al., 2013). Fifty species determined for genus *Enterococcus*, which they classified into different groups, based on *16S rRNA* sequence. Group D Includes; *E. casseliflavus*, *E. galinarium* and *E. flavus* (Gutierrez-Cortes *et al*., 2017).

They have been the subject of extensive studies in recent years because of their potential use as a novel, natural food preservative. During the fermentation of dairy products, these cultures metabolize lactose to lactic acid that, the low pH of fermented foods potentiates the antimicrobial effects of organic acids (Yerlikaya, 2014). These probiotics produce useful compounds such as bacteriocins, amino acids, vitamins, enzymes, and short-chain fatty acids, which provide health benefits for the host when consumed at appropriate dosages (Markowiak and Śliżewska, 2017).

The genus has also the potential to promote health by preventing the overgrowth of intestinal pathogens, aiding the development of mucosal immunity, enhancing secretion of mucin, prevention of intestinal inflammations and modulation of lactose intolerance (Kim *et al*., 2017).

Recently, there has been a growing interest in local food production and consumption, and consumers are looking for a substance suitable that go through the least processing (Verraes *et al*., 2014). Milk is a complex biological fluid and a pleasant growth environment for many microorganisms, the quality of milk is influenced by the microbial content in milk (Torkar and Teger, 2008). The camel is several purpose animals with massive productive potential. Camel milk is a key food in dried and semi-dried areas of the African and Asian countries. Nearly 150,000 dromedary camels are lived in desert areas (South and Central) of Iran. Camel milk production and consumption in Iran was limited to rural areas. Information on microbial quality, safety, and the food value chain of camel milk in Iran are confined (Abera *et al*., 2016). To increase people's awareness of the use of traditional food, beneficial effects of microorganisms and for application them in food processing as well as improvement and development of livestock in rural areas, therefore the objectives of the present study were to recognize the total bacterial content of raw camel milk in Torud village, in the Central District of Shahrud County, Semnan Province, Iran.

Materials and methods

Isolation of lactic acid bacteria

Ten samples of camel milk and Iranian traditional Doogh made from raw camel milk randomly collected from Semnan province of Iran. The regions under investigation were Shahrood city, Torud area. The samples were kept under aseptic conditions in a sterile flask and appropriate dilutions of samples plated onto MRS Agar. Plates incubated at 37 °C for 48 hours under anaerobic conditions (using Anaerocult ® a gas pack, Merck, Germany). Colonies with different morphologies counted and purified by re-streaking on the same medium. Gram staining, catalase test and microscopic observations performed for preliminary screening of *LAB*. The isolated *LAB* strains were analyzed by results of biochemical testing such as oxidase test, growth in 6.5% NaCl broth, hydrolysis of esculin in the presence of bile, pigment production, hemolytic activity, arginine hydrolysis, tolerance to tellurite, utilization of pyruvate, motility, resistance to vancomycin.

Molecular identification of lactic acid bacteria species

DNA extraction, *16S rRNA* gene sequence, and phylogenetic analysis

DNA was extracted with the Iranian Biological Resource Center and the primers used for the amplification of the *16s rRNA* genes (Macrogene, Korea). Table 1 shows primer sequences used and PCR condition for the amplification of the *16s rRNA* genes.

Table 1. Primers used and PCR condition

Gene	Primer sequences 5'_3'	Size of products(bp)	PCR conditions	Volume Re-actions
16s rRNA	5′-AGAGTTTGATYMTGGCTCAG-3′ 5′-CAKAAAGGAGGTGATCC-3′	1500	1cycle 94ċ.....13 min 30 cycle: 94ċ.....30s 58ċ.....30s 72ċ.....30s 1cycle: 72ċ.....8 min	10X PCR Buffer:2.5 µl 10 mM dNTPs: 0.5 µl 10 mM $MgCl_2$:0.75 µl 10 pmol F+R Primer: 1.25 µl Taq DNA polymerase (5u µl): 0.2 µl Template DNA: 1 µl H_2O up to 25 µl

The PCR products purified and sequenced by Macrogen Sequencing Service (Korea) and blast on NCBI. Finally, a Phylogenetic tree was constructed by using Clustal Omega.

Results

In this study, ten samples of camel milk and Iranian traditional Doogh made from raw camel milk randomly collected from Torud village, in the Central District of Shahrud County, Semnan Province, Iran. The Strains analyzed based on biochemical tests and morphological characteristics (Figures 1, 2). After that, *PslA* gene was identified in all of the strains using specific primers designed in all of the strains and its accuracy was determined with electrophoresis (Figure 1). The result showed all of the strains had *PslA* gene.

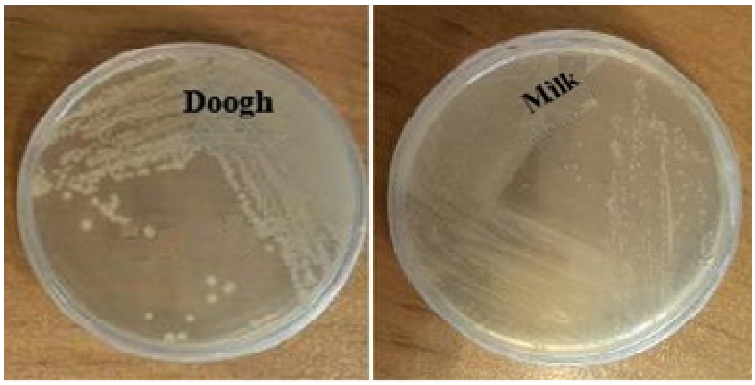

Figure 1. Cream or white colonies growing on MRS agar after 48 hours Incubated in CO2 at 37 ºC

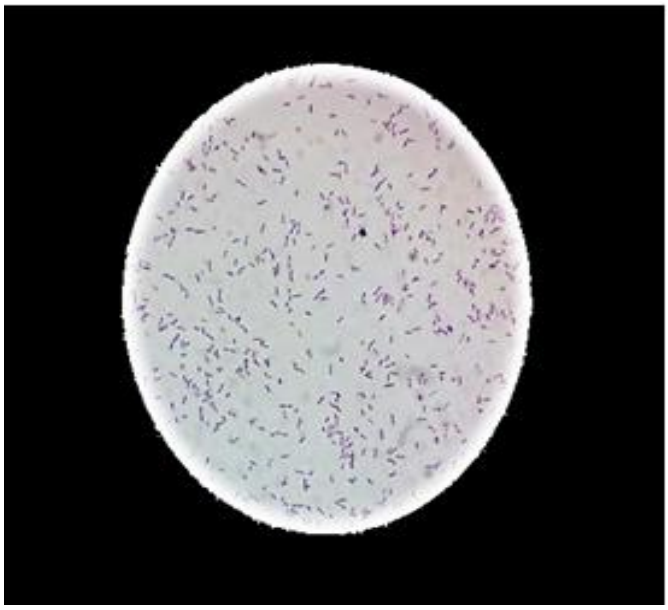

Figure 2. A smear of Gram's stained with gram-positive cocci bacteria in pairs (diplococci) or short chains, under a 100X light microscope

All these isolates were gram-positive, catalase-negative rods or cocci, as well as the examination of the observation, revealed that strains were vancomycin-resistant (Figure 3).

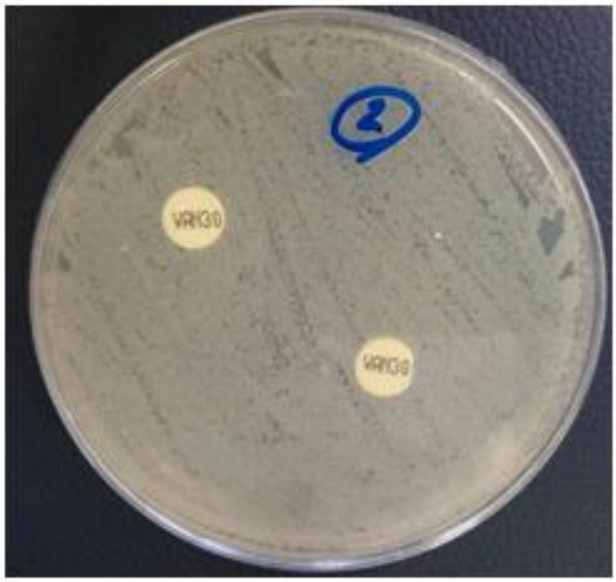

Figure 3. The antibiotics resistant to vancomycin (30 µg)

The frequently isolated were *Lactic Acid Bacteria* (*LAB*) and following that they identified as *E. gallinarum* and *E. casseliflavus* by ribosomal DNA sequencing (Figure 4).
These bacteria recorded with *E.gallinarum* strain STIMA MK578221 and *E. casseliflavus* strain STIBA MK580469 in the NCBI nucleotide database. The species relatedness of the *Enterococcus* phylogenetic tree is shown in (Figures 5A, 5B).

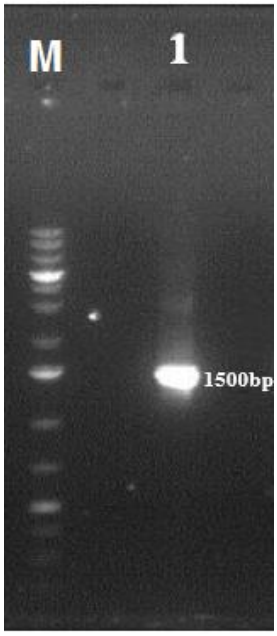

Figure 4. Agarose gel electrophoresis of the PCR products of two *Enterococci spp*. Isolated from Iranian row camel milk and Iranian traditional Doogh from raw camel milk (Semnan province, Shahrood city, Torud area), M. 1 Kb marker 1. Sample gene

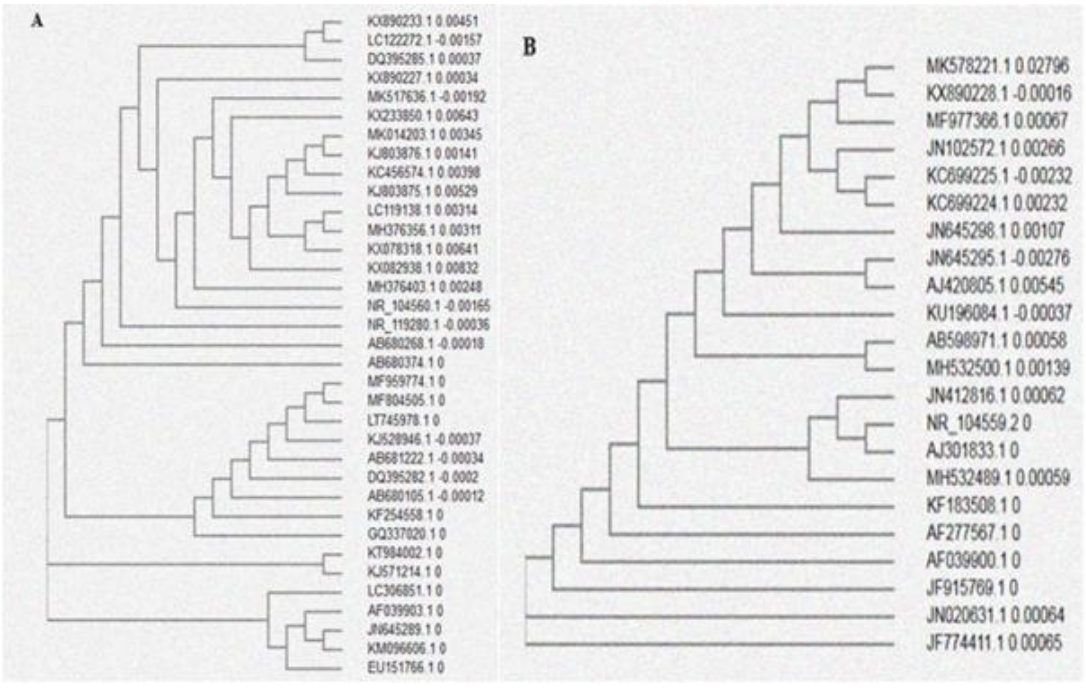

Figure 5. Phylogenetic tree based on *16S rRNA* gene sequences showing base homology of, A. The isolate *E. casseliflavus* and, B. The isolate *E. gallinarum*

Discussion

These results are in agreement with the findings of some other researchers such as: In Baghdad, the *Enterococcus* spp. Identified in samples of raw cow milk and vaginal swabs

from aborted women and patient women by microbial and biochemical tests. The major *Enterococcus* species of raw milk samples were *E. faecalis*, *E. durans*, and *E. gallinarum* (Hamzah *et al.*, 2018). A study in 2015, was done for isolation and molecular identification by *16S rRNA* sequencing of *LAB* from camel's milk and evaluation of their probiotic properties in the Golestan province of Iran; the most commonly isolated *LAB* was *enterococci*, while others *lactobacilli* were found less frequently (Davati *et al.*, 2015).
In another study, investigating species distribution and antimicrobial effects among food *Enterococci* were studied in Rio de Janeiro. The species such as *E. casseliflavus*, *E. durans*, *E. gallinarum*, from hen meat and pasteurized milk were isolated (Fracalanzza *et al.*, 2007). In another study, the genus *Enterococcus* spp. were isolated and identified from nonpasteurized milk samples obtained from the Costa Rican Metropolitan area, *E. faecalis*, *E. faecium*, *E. gallinarium*, *E. durans* and *E. avium* were isolated. All isolates showed the highest resistance to vancomycin which same like to our results (Araya *et al.*, 2005).
Another same finding showed *E. casseliflavus*, *E. faecalis*, and *E. durans* exist in raw milk cheeses (Gelsomino *et al.*, 2002). In Italy, 124 enterococcal as *E. faecium* (27 strains), *E. faecalis* (82 strains), *E. gallinarum* (four strains) and *E. hirae* (two strains), *E. durans* (nine strains) were isolated from buffalo and goat cheeses by biochemical and RAPD–PCR methods (Andrighetto *et al.*, 2001). The other study in Italy, *E. gallinarum*, *E. faecalis*, *E. durans*, and *E. hirae* isolated from a traditional cheese. They were a remarkable portion of the microbial crowd of this cheese. Hence, confirmed the significance of the presence of these micro-organisms during the cheese-making process. (Suzzi *et al.*, 2000). Two kinds of special sheep cheese in Spain have high levels of *Enterococcus* bacteria. Eighty-five percent of *Enterococci* spp. were *E. faecalis*, *E. faecium*, *E. durans* and *E. avium* in these kinds of cheese (Arizcun *et al.*, 1997). Likewise, (Naceur *et al.*, 2016) isolated 23 *Enterococcus* spp. from 33 samples of camel's milk collected from the southwest region of Algeria. In a survey, 92 isolates of *LAB* utilizing frozen camel's milk was identified. Of these, 44.56% and 55.43% were determined to be as rods and cocci isolates, respectively (Ashmaig *et al.*, 2009).
Our study showed the species relatedness of the *Enterococcus* phylogenetic tree by using *16S rRNA* gene sequences which is in agreement with the study by (Zhong *et al.*, 2017) who showed Phylogenetic tree of 49 *Enterococcus* type strains based on *16S rRNA* sequences that the maximum likelihood tree with *Lactobacillus casei* ATCC 334, *Lactobacillus plantarum* WCFS1 and *Lactobacillus rhamnosus* ATCC 8530.

Conclusion

This study showed that camel's milk is a rich source of probiotic bacteria. The isolation and use of these bacteria in dairy products are effective in protecting the health of consumers. Hence, these bacteria could be a strong candidate for using in food and pharmaceutical industry.

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