



## COMPARATIVE CHARACTERIZATION OF LACTIC ACID BACTERIA ISOLATED FROM DONKEY'S MILK

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### ABSTRACT

It was shown that the strains of LAB which isolated from milk of different domestic animals (donkey) of Republic of Armenia and Republic of Artsakh have differ species and genus affiliation, as well as different physiological, antibacterial and probiotic properties. Cultural liquid was purified by method of gel filtration. Antimicrobial preparations (AMP) were obtained from culture liquids, which had different efficacy inhibition the growth of antibiotic-resistant strains. The selected strains are mainly represented by the genus of Enterococcus and may have perspective for further as biopreservation in food industry.

**KEYWORDS:** Endemic lactic acid bacteria, donkey's milk, probiotic, AMP.

### INTRODUCTION

In the last years, a lot of attention is paid to the probiotic properties of LAB which isolated from traditional dairy products. It is considerably that in a number of European countries a great attention is paid to the probiotic and antimicrobial properties of LAB which isolated from donkey's milk.<sup>[2]</sup> Interest in donkey's milk has significantly increased, largely due to the irreplaceable functional elements contained in milk. Even if it's known to be a donkey's milk ingredient and nutritional properties, microflora is less studied. Donkey's milk is rich in microbes that can be a great source of probiotics, for example, containing *Lactobacillus rhamnosus* LAB strains.<sup>[7]</sup>

For the several strains which isolated from donkey's milk from Northwestern farms in Italy was used identification RAPD-PCR and 16S r-RNA gene expression. The results have shown that the most frequently encountered species are *Lactobacillus paracasei*, *Lactococcus lactis* and *Carnobacterium maltaromaticum*, less *Leuconostoc*, *Enterococcus*, *Streptococcus*, and *Kluyveromyces marxianus*.<sup>[12]</sup>

Identification data of the isolated lactic acid bacteria isolated from Ragusana, the type of substance (16 R-RNA segregation), showed that the majority of LAB strains belong to *Lactococcus lactis subsp. Lactis*, *cremoris*, and *Lactobacillus paracasei*. These lactic acid bacteria have coagulating and acidifying properties, 50% of which increase with 5.0 g / L of lysozyme. About 70% of isolated yeasts are attributed to the *Kluyveromyces lactis* species.<sup>[15]</sup>

Many studies have clarified that donkey's milk microbiota is focused to detect pathogenic bacteria.<sup>[8,14]</sup> Donkey milk is considered an interesting substitute to cow's milk thanks to its nutritional properties. The chemical composition of donkey milk has been extensively considered essential to prevent the growth of undesirable microorganisms and few data are available on its microbiological characterization. In the present study raw and cultured donkey milk samples were analyzed in order to characterize lactic acid bacteria. Lactic acid bacteria isolated strains were identified using a combination of different molecular techniques and results were confirmed by sequence analysis of the 16S rRNA gene. Furthermore, technological traits of the strains were investigated. The results indicated that dominant lactic acid bacteria strains were identified mainly as *Lactococcus lactis* subsp.<sup>[8,14, 17,4]</sup>

LAB content of donkey milk range between 1.0-4.2 log cfu/ml, but only few LAB isolates have been identified.<sup>[7,5, 19,16]</sup>

Fresh donkey's milk is a natural source of probiotic bacteria that are able to colonize the gastrointestinal tract, fighting against pathogenic bacteria, stimulating the immune system. It contains lipolytic enzymes that break down the fatty molecules in the gastrointestinal tract. It is rich in immunoglobulins and is considered a natural antibiotic.<sup>[6]</sup>

Based on the above mentioned, it was interesting to study some probiotic properties of the LAB's which isolated from the donkey's milk from Artsakh Republic (AR) and compare

them with LAB's, which were isolated from donkey's milk from Republic of Armenia(RA).

#### MATERIALS AND METHODS

**Microbial strains and growth media.** The endemic LAB cultures were isolated from donkey's milk of Republic of Armenia (RA) and Artsakh Republic (AR). Samples were collected in sterile small bottles and stored at 4°C in the laboratory until they were used in experiments. Serially diluted samples were spread on MRS agar (Merck, Germany) and hydrolyzed milk agar (1,2% w/v) and cultivated at 37°C. Different morphotypes of colonies were selected and obtained pure cultures were characterized according to standard methods for lactic acid bacteria. Pure cultures of LAB were maintained as frozen stocks at -20°C in the MRS broth containing 40% Glycerol. Before use, they were transferred twice into the appropriate medium and incubated during 48 hours in temperature controlled conditions in thermostat at 37°C.

**Inoculum preparation and obtaining of cell-free culture broth.** Single colonies were grown in five ml of MRS broth (37°C, 24 h) and when were transferred into 100 ml-Erlenmeyer's flask containing 50 ml of MRS broth and incubated overnight at 37°C for 24 hours in the thermostat. 50 ml of the obtained inoculum was transferred into 800 ml of MRS broth in 1L Erlenmeyers flask and grown at 37°C for 48 hour in the thermostat. At the end of growth culture broth centrifuged at 6,000 rpm during 20 min and obtained cell free culture broth (CFC broth) was used for future purification.

**Purification of the CFC broth.** Cell free culture broth of LAB strains was purified by combination of adsorption-desorption and ion-exchange chromatography methods.<sup>[1]</sup>

**Sensitivity of LAB to bile and pH, enzymes.** The isolated strains of LAB were incubated in a nutrient media MRS broth (Merck) with content of definite amount of bile during 24 hours at 37°C in a thermostat. Survival of LAB in the conditions close to those as in intestine (influence of digestive enzymes, pH in the range 3.0-8.0 was checked according to the generally accepted method [9]. There were used the following chemical agents in the work: Trypsin, Pure from bovine pancreas 3x, activity 2500 NFU/mg (HIMEDIA), Pepsin, Extra pure (1:3000), (HIMEDIA). Survival of the LAB was estimated by the change of the optical density at 590 nm.

**Determination of resistance to antibiotics.** To determine the resistance of isolated pathogens to antibiotics, the method with standard antibiotic disks (Oxoid, UK) was applied.<sup>[3]</sup>

**Test cultures and determination of antimicrobial activity.** To determine antimicrobial properties of samples, conditionally pathogenic bacteria from the culture collections of Department of Center of Microbial Depository (CMD) and Laboratory of Food Safety of SPC "Armbiotechnology" were used. Antibiotic resistant pathogenic bacteria strains pathogenic bacteria, isolated from various physiological fluids of infected human patients, were investigated in the Stepanakert Center for Hygiene and Epidemiology. Bacteria were grown in Nutrient agar (Himedia, India) at pH 7.2 for 16 hours and at 37°C, then harvested and suspended in the Nutrient broth at  $2.2 \times 10^6$  CFU/ml. For determination of antimicrobial activity the spot-on-lawn method on the test culture inoculated in the solid medium was applied. The antimicrobial activity is expressed in arbitrary units (AU ml<sup>-1</sup>).

#### RESULTS AND DISCUSSION

Previously, we were published the results of studies on the selection of strains with probiotic properties from different samples of milk and dairy products from different domestic animals.<sup>[11]</sup> It was interesting to compare probiotic activity with the LAB which isolated from differ donkey's.

We was isolated LAB's from donkey's milk from different regions of Artsakh and RA . The strains were respectively designated AV -3, 2- AV -5, 3-AV-9, from Armenia 4-KE1, 5-KE2, 6-KE3, 7-KE4, 8-KE5, 9-KE6, 10-KE7, 11-KE8, 12-KE9, 13-KE10 from Artsakh. The probiotic properties were evaluated for a number of features.

We have studied the viability of isolated LAB's to trypsin and pepsin enzymes. The results show that the viability of LAB's, which isolated from donkey's milk from Armenia is about 30% for enzymes, while the viability of LAB's which isolated from donkey's milk from Artsakh is 80% higher (Fig.1).

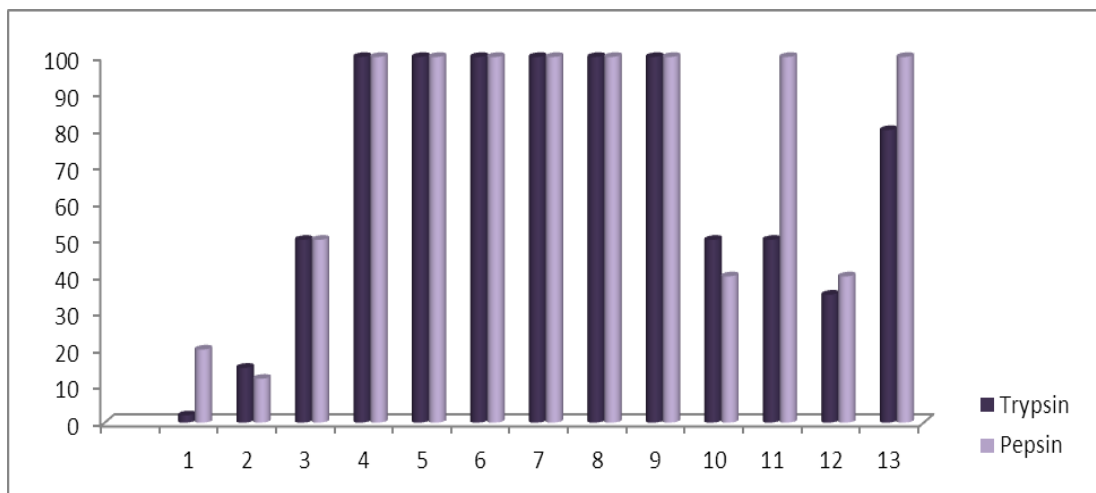


Fig. 1: The viability LAB's to enzymes, %.

From the data on the stability LAB's to antibiotics, it became clear that the LAB's isolated from Artsakh's donkey's milk are stable against the most commonly

used antibiotics (gentamicin, ofloxacin, ciprofloxacin) in medicine, whereas LAB's which isolated from Armenian donkey's milk are sensitivity to antibiotics (Fig.2).

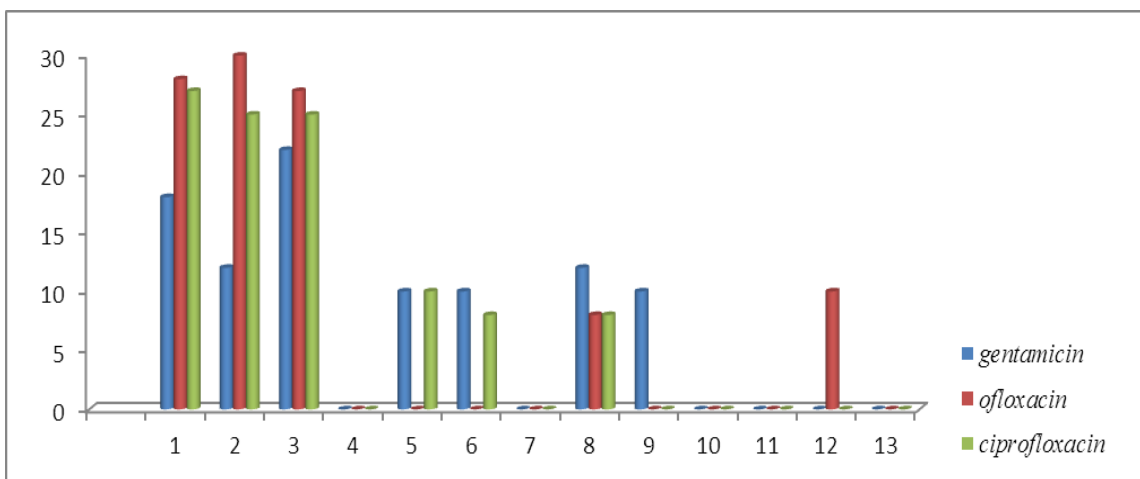


Fig. 2: The sensitivity LAB's to antibiotics, Ømm.

One of the most important probiotic properties of LAB's is the viability to bile. The viability of all LAB's is 35-50%, with the exception of LAB sp. which have 90%

viability in the range 0.2-0.5% and 55% in the range 0.5-0.8% of bile.

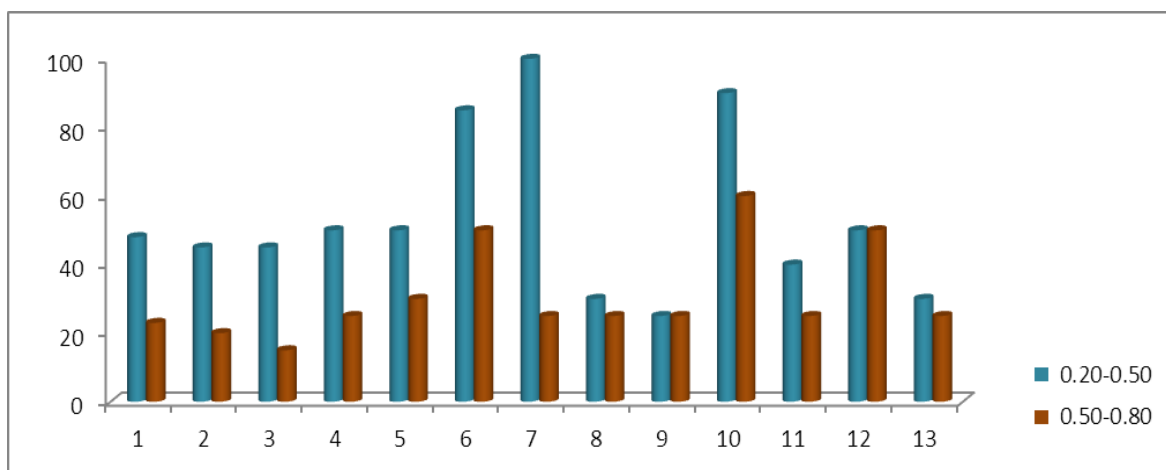


Fig. 3: The viability LAB's to bile, %.

We investigated the viability of the isolated LAB's in pH 2-9 pH ranges. It was shown that the LAB's which isolated from donkey's milk are more viable in the baseline pH than LAB's which isolated from different domestic animals. We was comparing LAB's which isolated from Artsakh's donkey's milk with the LAB's

which isolated from Armenian donkey's milk. It was shown that the LAB's which isolated from Artsakh's donkey's milk are viable in the wide pH range than the LAB's which isolated from the Armenian donkey's milk (Fig.4).

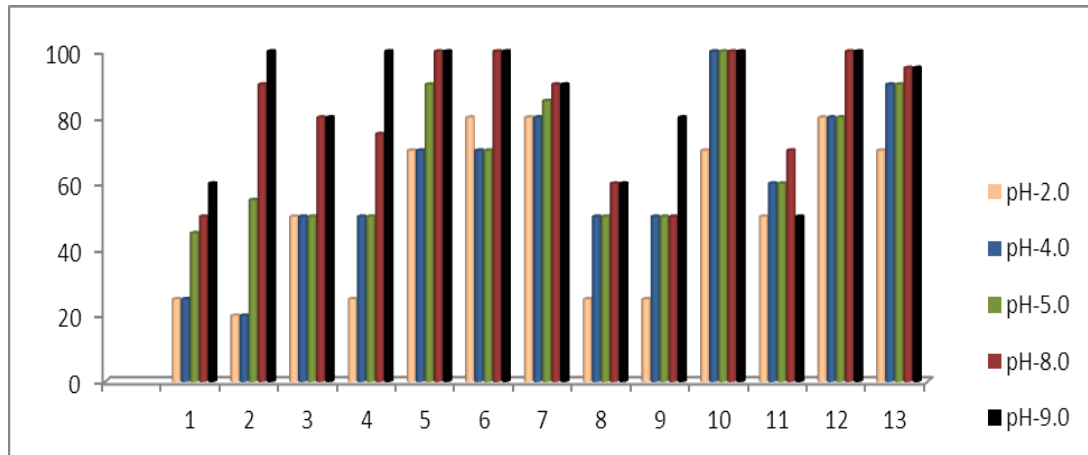


Fig.4: The viability LAB's in pH ranges, %.

By the method of ion-exchange chromatography was developed by us, was obtained partially purified antimicrobial bio-preparations (AMP).<sup>[1]</sup> The investigation of antimicrobial activity of AMP against conditionally pathogenic bacteria and multidrug resistant

human pathogens are presented in the Figure 5. As it seen from the given results, AMP of different LAB strains inhibited the growth of pathogenic bacteria with different efficiency.

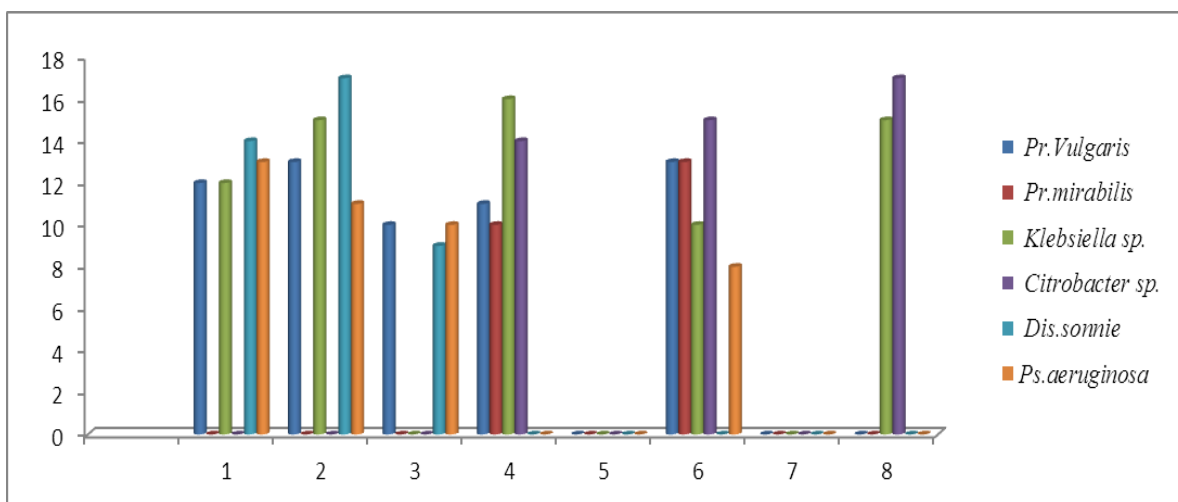


Fig 5: Comparative antimicrobial activity of AMP against bacteria of different taxonomic groups, Ømm.

Average statistical results proved that strains of LAB shown less efficiency against antibiotic resistant bacteria in comparison with conditionally pathogenic bacteria. The diverse efficacy of growth inhibition may probably relate to the different mechanisms of action of the substances towards bacteria cell membrane. The investigation of adhesion on epithelial cells and antioxidant activity of strains showed that the selected strains from Artsakh had a high degree of activity. Comparison of the obtained data with the data obtained by us on the probiotic properties of strains isolated and examined from milk and dairy products of cows show to the perspective of using strains

from donkey's milk.<sup>[10]</sup> The results of genotyping (16 S RNA) showed that the strains (KE5-Enterococcus durans, KE6-Enterococcus durans, KE9-Enterococcus faecium, KE10-Enterococcus faecium.) which isolated from Artsakh donkey's milk are mainly represented by the genus of Enterococcus. The strains (A1, A2, A3) isolated from the donkey's milk of the different region of Armenian represented by the genus of Lactobacillus. The strains are deposited in the Museum of the Depository Center of the Scientific and Production Center "Armbiotechnology".

The obtained data indicate that the ecological and geochemical isolation of the Republic of Artsakh, its geographical location, the absence of a negative influence of the anthropogenic factor, have become the basis for the creation and maintenance of endemic LAB's that can be promising and can be used as basis for obtaining the new products of functional nutrition with antimicrobial and health promoting effects. Perspective of the use of certain AMP from lactic acid bacteria against pathogenic strains is obvious. These AMP can be applied for long term use against different etiology antibiotic resistant pathogens for prevention or treatment of infectious diseases as an alternative to antibiotics.

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