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Novel lactic Acid Bacteria in raw cow's milk from highland farms

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ABSTRACT

Lactic Acid Bacteria (LABs), a member of probiotic bacteria play important roles in everyday life world-wide. In an effort to explore the possibility of isolating novel LABs, raw milk samples were collected from Alborzmountain regionsin the north of Iran.Primary investigations were performed to evaluate the probiotic potentials of the isolated strains. After characterization, isolates were categorized in 9 groups by High Resolution Melting Real-Time 16S rDNA PCR. Indicator strains of each group were identified by PCR-Sequencing and the presence of Glutamate Decarboxylase Gene was also investigated as a prominent probiotic characteristic. Three potential novel species; Pediococcus shahsavar, Enterococcus alborz and Pediococcus siga1and also a novel strain of Enterococcus faecalis (rudsar) were identified, and their sequences were submitted to NCBI. This studyshows the presence of high diversity of LABs in the raw milk of Iran Alborz mountain regions that warrants further research in this area.

Keywords: highland farms, raw cow's milk, Lactic Acid Bacteria

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INTRODUCTION

Lactic Acid Bacteria (LABs) as a major group of gram positive, catalase negative bacteria are the most important constituent of probiotics and have numerous applications in industry [1]. This large group of bacteria includes more than 20 genuses to date [2]. LABs are mostly microaerophilic and produce lactic acid as their major final product from fermenting carbohydrate [3]. Although these bacteria are fastidious but are isolated from various sources [4]. Among the most important sources are milk and its products. LABs are responsible for milk aromatic and physiochemical conversion to dairies [5]. Because of their high biotechnological potential, isolation and characterization of new strains of LAB for broader industrial application has always been of great importance [1].

Today, application of a combined polyphasic method using phenotypic and genotypic techniques is the best method for LABs identification [6]. Sometimes morphological observation with some routine tests would be enough to identify an LAB while in other situations a technique capable of identifying a particular strain might not distinguish between two species [7]. In recent years, molecular biology techniques have been increasingly used for identification of LABs [8].

The aim of this study was to isolate, identify and characterize potential novel strains of LABs from raw milk samples. We also investigated the potential probiotic features of isolated LAB strains. To achieve our goals a combination of morphologic, physiologic and molecular methods were applied. Due to the influence of flora on the diversity of LABs in raw milk [9], the traditional herds from unique Central Alborz Mountain regions in north of Iran were selected for sampling.

MATERIAL AND METHODS

2. 1. Sampling

Raw milk samples from 14 selected areas of Central Alborz Mountain regions north of Iran at altitude ranging from 1000m to 3000m above sea level were collected and transferred to the laboratory and stored at 4ºC.

2. 2. Enrichment

Raw milk samples were first homogenized by vortexing and then,10ml from each sample was transferred to 90ml of MRS and M17 broth media (Merck, Darmstadt, Germany) and incubated at 30ºC for 48h with 10% CO2 (memmert, Schwabach, Germany) [10].

2. 3. Primary Isolation

The enriched samples were serially diluted [11] and dilutions of 10^{-4} to 10^{-8} from MRS medium and 10^{-5} to 10^{-8} dilutions from M17 medium were inoculated to plates with spread culture and incubated in anaerobic jar containing Anaerocult C (Merck, Darmstadt,Germany) at 30°C for 48h [6]. Morphological observation, Gram staining and catalase activity test were performed [12] and gram positive, catalase negative colonies considered as probable LABs were sub-cultured twice for purification [6].

2. 4. Phenotypic Characterization

Isolated colonies were separately incubated at 37ºCand 45ºC for 24h to determine the optimal temperature of the strains. After primary phenotypic characterization, acid and bile tolerance (two basic properties of Probiotics) of the isolates were studied [8].

2. 4. 1. Acid Tolerance

A combination of methods with some modifications were used for primary testing of acid tolerance of the isolates [13]. 100µl of broth medium from each isolate was transferred in 2ml of acidic phosphate saline buffer solution (pH= 2.5) and incubated in CO2 incubator (10% CO2) at 37°C for 2.5h. Afterwards, samples were centrifuged for 20 min. at 5000 rpm, washed with phosphate saline buffer (Ph. 7.0) and cultured on the appropriate solid media. Cultured plates were placed in anaerobic jar containing Anaerocult C and incubated for 24h at 37°C for isolation of acid tolerant strains.

2. 4. 2. Bile Tolerance

To determine the ability of strains to grow in the presence of bile, we used the methods previously described by Vinderola et al. [14] and Pereira et al. [15] with some modifications. 1% (v/v) broth culture of each isolate was inoculated in broth medium containing
0.3% bile (OXBILA, QUELAB, Montreal, Canada) and incubated at 37ºC in the presence of 10% CO2 for 24h. A_{600nm} of each isolate and its control culture (without bile salts) were measured and samples with A_{600nm}> 50%were considered as bile tolerant.

2. 5. Molecular Surveys

2. 5. 1. DNA Extraction

DNA from all the isolates were extracted by using a commercially available kit (Roche, Basel, Switzerland) and stored for further molecular examinations after quality and quantity control [16].

2. 5. 2. High Resolution Melting Real-Time PCR

High Resolution Melting Real-Time PCR (HRM Real-Time-PCR) was performed as the primary molecular method [17]. DNA samples from all the isolates were amplified and categorized into groups by analyzing HRM (Corbett, RG6000).

Each PCR reaction tube contained 50-100ng of each DNA template, 5µl 10X buffer, MgCl2 1.5mmol, dNTPs 0.2mmol, 10pmol of each primer, 2.5 U of Taq DNA polymerase (Fermentas, Burlington, Canada) and 10µmol SYTO-9 (Invitrogen, USA) in a total volume of 25 µl. The following primers used were for HRM-Real-Time PCR; 16S rDNA universal primers DG74 (5'-AGGAGGTGATCCAACCGCA-3') and RW01 (5'-AACTGGAGGAAGGTGGGGAT-3') [18].

HRM-Real-Time PCR conditions consisted of a pre-denaturation at 95°C for 4min followed by 35 cycles of 95°C for 40sec, 56°C for 30sec, 72°C for 40sec and a final extension of 72ºC for 5 min. HRM was analyzed in 72°C-90°C with a 0.1°C temperature shift in 1 second and absorption in Green Channel. The PCR products were analyzed on a 2% agarose gel and confirmed by presence of 380 bp band.

2. 5. 3.16S rDNA PCR-sequencing

To obtain sequencing quality DNA for molecular identification, PCR reactions were performed on indicators and one or two randomly selected members of each group in MyCcler PCR machine (Bio-RAD, CA, USA). PCR conditions were the same as HRM Real-Time PCR (50µl total volume and 40 cycles) and contained all the ingredients except SYTO-9. After qualitative confirmation of amplicons, sequencing was performed (Macrogen, Seoul, Korea). Resulted sequences were aligned with National Center for Biotechnology Information (www.ncbi.nlm.nih.gov) Gene Bank sequences using online Blast software to identify the isolates.

2. 5. 4. Glutamate Decarboxylase Gene PCR

The presence of Gamma Aminobutyric Acid (GABA) producing gene (*GAD*) was examined in all samples by PCR. Each PCR reaction consisted of 50-100ng of DNA, 200µmol of each dNTP, 1µmol of each primer, 2 µmol of MgCl2, 2 U of Taq DNA polymerase (Fermentas, Burlington, Canada) and 5µl of 10Xbuffer in a total volume of 50µl.

The following PCR primers; Core F (5'-CCTCGAGAAGCCGATCGCTTAGTTCG-3') and Core R (5'-TCATATTGACGGGTATAAGTGATGCCC-3') were used to amplify the 540 bp target sequence[19]. The PCR conditions were: pre-denaturation at 95°C for 4min followed by 40 cycles of 95°C for 45sec, 55°C for 30sec, 72°C for 1min and a final extension of 72°C for 5min.

PCR products were assessed by 1.5% (wt/vol) agarose gel electrophoresis and the presence of 540 bp DNA fragment confirmed *GAD* positive strains.

RESULTS

3. 1. Phenotypic Characterization

After primary enrichment 164 colonies were randomly selected from M17 and MRS media. From those, 62 gram positive catalase negative colonies were considered as probable LABs. After purification, phenotype characteristics, and resistance of the isolates to acid and bile were studied and the results are shown in Table 1.

Table 1. Phenotypic Characterization Results of Isolates

Sample Designation	Morphology	Growth in 30°C	Growth in 37°C	Growth in 45°C	Acid Tolerance	Bile Tolerance
S1A4	Cocci	✔	✔	✔	✔	✔
S4A4	Cocci	✔	✔	✔	✔	✔
S3A4	Cocci	✔	✔	✔	✔	✔
S6A10	Cocci	✔	✔	✔	✔	-
S9A12	Cocci	✔	✔	-	✔	-
S37A2	Cocci	✔	✔	-	✔	-
S13A8	Cocci	✔	✔	✔	✔	✔
S8A12	Cocci	✔	✔	✔	✔	✔
S12A13	Cocci	✔	✔	✔	✔	✔
S11A1	Cocci	✔	✔	✔	✔	✔
S61A12	Cocci	✔	✔	-	✔	✔
S28A8	Cocci	✔	✔	-	✔	✔
S16A6	Oval	✔	✔	-	✔	✔
S60A8	Cocci	✔	✔	-	✔	✔
S19A5	Cocci	✔	✔	✔	✔	✔
S21A5	Oval	✔	✔	✔	✔	✔
S48A4	Cocci	✔	✔	✔	-	✔
S59A4	Cocci	✔	✔	-	-	✔
S20A5	Cocci	✔	✔	✔	✔	✔
S23A4	Cocci	✔	✔	✔	-	-
S24A4	Cocci	✔	✔	-	-	-
S49A4	Cocci	✔	✔	✔	-	-
S53A2	Rod	✔	✔	-	-	✔
S57A4	Rod	✔	✔	-	-	-
S58A4	Rod	✔	✔	-	-	-
S25A4	Rod	✔	✔	-	-	-
S26A4	Rod	✔	✔	-	-	-
S29A7	Cocci	✔	✔	-	✔	✔
S5A11	Cocci	✔	✔	-	✔	-
S7A9	Cocci	✔	✔	✔	✔	✔
S10A1	Cocci	✔	✔	✔	✔	-
S27A8	Cocci	✔	✔	-	✔	-

S30A7	Cocci	✔	✔	-	✔	✔
S44A5	Cocci	✔	✔	-	✔	-
S43A5	Cocci	✔	✔	✔	-	-
S14A10	Cocci	✔	✔	-	✔	-
S35A1	Cocci	✔	✔	-	✔	-
S38A1	Cocci	✔	✔	-	✔	-
S31A6	Cocci	✔	✔	-	-	-
S45A5	Cocci	✔	✔	-	-	-
S46A5	Cocci	✔	✔	✔	-	-
S36A1	Rod	✔	✔	-	✔	-
S39A14	Rod	✔	✔	✔	✔	-
S63A9	Rod	✔	✔	-	-	-
S52A1	Rod	✔	✔	-	✔	-
S64A10	Rod	✔	✔	-	-	-
S42A13	Rod	✔	✔	-	✔	✔
S54A1	Rod	✔	✔	-	✔	-
S51A4	Cocci	✔	✔	-	-	✔
S2A4	Cocci	✔	✔	✔	✔	✔
S15A5	Cocci	✔	✔	✔	✔	✔
S17A5	Cocci	✔	✔	✔	✔	✔
S22A5	Cocci	✔	✔	✔	✔	✔
S18A6	Cocci	✔	✔	✔	✔	✔
S47A4	Cocci	✔	✔	-	-	-
S56A4	Cocci	✔	✔	✔	-	✔
S55A3	Cocci	✔	✔	-	-	✔
S62A2	Cocci	✔	✔	✔	-	✔
S66A11	Cocci	✔	✔	✔	-	✔
S40A14	Rod	✔	✔	✔	✔	✔
S41A13	Rod	✔	✔	-	✔	✔
S65A10	Rod	✔	✔	-	✔	✔

3. 2. High Resolution Melting Real-Time PCR

Based on the HRM-RT PCR results, samples were divided into 9 groups (A-I). Real Time results were all confirmed by presence of 380 bp amplicons (Figure 1).

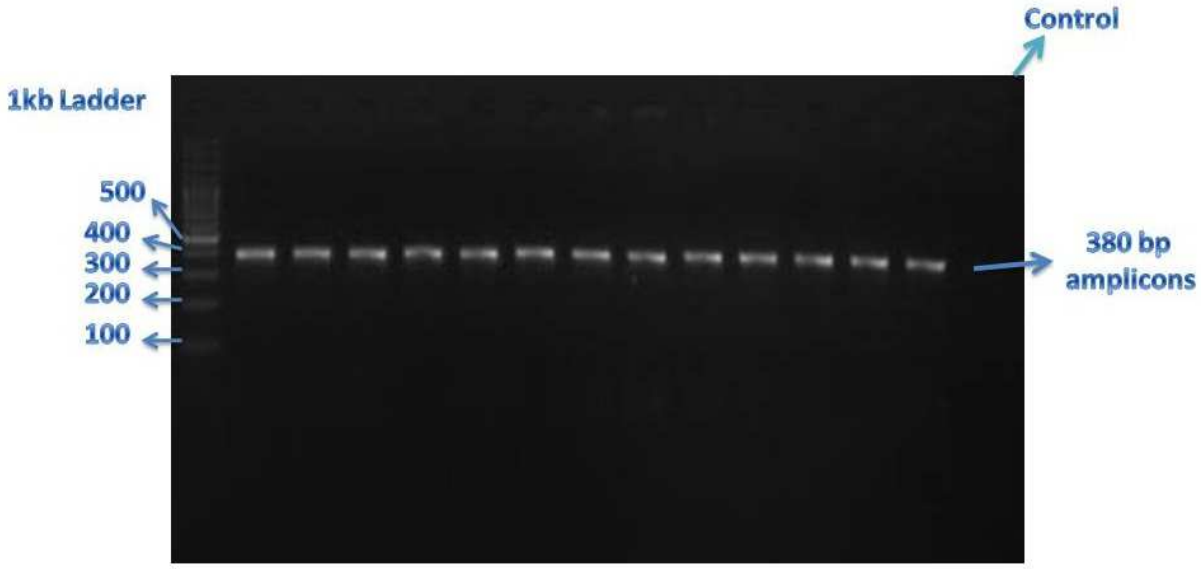

Fig. 1. A 2% agarose gel showing the presence of 380bp PCR amplicons and a negative PCR control.

Geographical distribution of strains was diverse and except sample S1A4 the rest were isolated from more than one area (Table 2). Comparing the results from HRM-RT PCR with microscopic examination revealed that, groups F and G consisted of both spherical (F1/G1) and rod (F2/G2) bacteria. Therefore beside the indicator strains and randomly selected strains from all the groups for 16SrDNA PCR sequencing, more samples of each morphological subgroup from groups G and F were also picked for sequencing.

Table.2 Abundance and Geographical Distribution of Isolates

					Groups by HRM Analysis					
A	**B**	**C**	**D**	**E**	**F**		**G**		**H**	**I**
					F^1	**F^2**	**G^1**	**G^2**		
S1A4	**S4A4**	**S8A12**	**S11A1**	**S19A5**					**S51A4**	**S40A14**
	S3A4	**S12A13**	**S61A12**	**S21A5**	**S20A5**	**S53A2**	**S29A7**	**S36A1**	**S2A4**	**S41A13**
	S6A10		**S28A8**	**S48A4**	**S23A4**	**S57A4**	**S5A11**	**S39A14**	**S15A5**	**S65A10**
	S9A12		**S16A6**	**S59A4**	**S24A4**	**S58A4**	**S7A9**	**S63A9**	**S17A5**	
	S37A2		**S60A8**		**S49A4**	**S25A4**	**S10A1**	**S52A1**	**S22A5**	
	S13A8					**S26A4**	**S27A8**	**S64A10**	**S18A6**	
							S30A7	**S42A13**	**S47A4**	
							S44A5	**S54A1**	**S56A4**	
							S43A5		**S55A3**	
							S14A10		**S62A2**	
							S35A1		**S66A11**	
							S38A1			
							S31A6			
							S45A5			
							S46A5			

3. 3. Molecular Identification

All of the 62 isolates were identified at genus/species level by aligning of our 16SrDNA PCR sequences with those from NCBI gene bank. Sample S1A4 as the only member of group A showed 78% homology with *Pediococcusacidilactici* and was submitted as a potential new species *P.siga1* (Accession Number: HQ823617).

Members of group B were identified as Weissella after aligning results of the group indicator (S4A4) with one randomly selected member, showing 99% of homology with *W.paramesenteroides*.

Members of group C were identified as *Enterococcus faecalis* and the group indicator (S8A12) was submitted as a potential new strain *rudsar* (Accession Number: HQ646363).

Members of group D, and group E were identified as *Lactococcus lactis* and *Streptococcus infantarius*, respectively. Indicator strains of both groups showed 100% homology with gene bank reference sequences.

Polyphasic examination of groups F and G showed the presence of both rod and spherical bacteria. Therefore16SrDNAs of 2 indicator strains from each subgroup of group F, and 3 indicator strains from each subgroup of group G were sequenced. Accordingly *Leuconostoc argentinum*(F1) and *Lactobacillus brevis*(F2) were identified in group F and *Pediococcus pentosaceus*(G1) and *Lactobacillus rhamnosus*(G2) in group G. Beside identification of group F and G members at genus/species level, one strain (S31A6) was submitted as probable new species *Pediococcus shahsavar* (Accession Number: HQ705607).

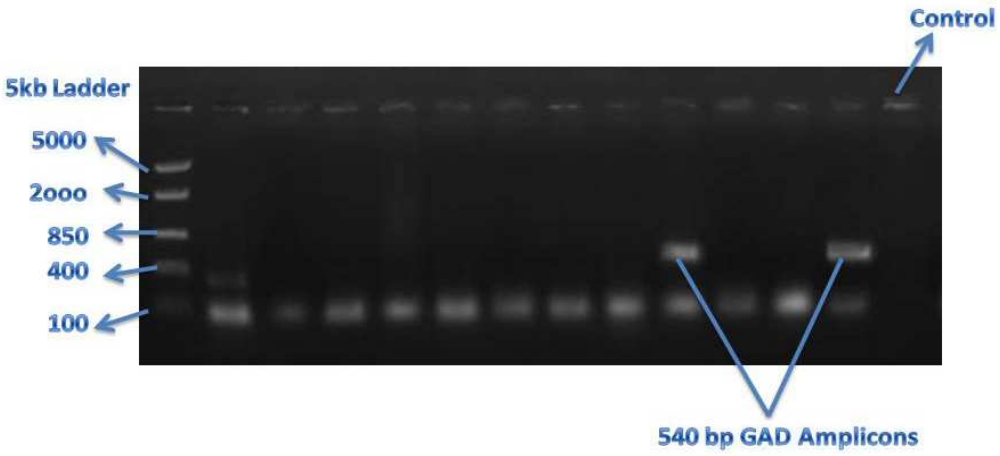

Fig. 2. Shows the presence of 540bp PCR amplicons from GABA producing gene (*GAD*)from two isolates and a negative PCR control on a 2% agarose gel.

Samples of group H were identified as genus *Enterococcus* while an indicator strain of the group (S51A4) was submitted as a potential new species *E.alborz* (Accession Number: HQ873640). Finally, members of group I were identified as *Lactobacillus plantarum.*

3. 3. Glutamate Decarboxylase Gene

The *GAD* gene PCR results showed that, all 3 *L.plantarum* isolates, 3 of *L.rhamnosus* isolates (S39A14, S42A13, S63A9) and one *Lactococcus lactis isolate*(S11A1) possessed GABA producing gene (Figure2).

DISCUSSION

Modern applications of LABs have a long history in developed countries. In the past two decades, importance of these bacteria in industry and health improvement has encouraged other countries to make serious efforts to isolate and identify their local LABs, and optimize them for industrial applications. Madoroba and colleagues [20] in South Africa, Sujaya and colleagues [21] in Japan and many other scientists around the world have been recently working on LABs. In the present study, our goal was to achieve a primary identification of local LABs present in raw milk of Alborz mountain regions in the north of Iran and also to evaluate their industrial potentials.

Since the climate, flora and environment of Central Alborz Heights are very different from other regions and considering the effect of flora on LABs diversity in milk [22] traditional herds of this area were selected as sampling source. Lack of any previous studies and reports from these regions suggested a high possibility of isolating novel LABs.

Simultaneous application of HRM RT-PCR with about 80% confidence level for grouping of isolates and along with 16S rDNA PCR sequencing resulted in a faster and cheaper identification of strains. The application of HRM RT-PCR showed to be an appropriate method for our studies. Only in two groups (F and G), the HRM was unable to differentiate between the two genuses due to, high similarity of melting properties in partial 16S rDNA sequences. Based on the percent homology existing between isolates, one can conclude that, identification based on sequencing results would be certain at species level for indicator strains of the groups and at genus level for all other isolates[23].

Although the observed homology between the isolated LABs with the reference strains indicates the presence of potential novel species, but we believe that, our findings are preliminary and require further confirmation by sequencing more relevant genes such as *gyrA, tuf, rpoB* and others as well [24].The prevalence of high diversity among the isolated LABs, industrial importance of these strains and some of their probiotic potentials such as the presence of *GAD* gene in a strain of *l.lactis* are important findings of this study.

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