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Lactobacillus plantarum A7, a Potential Probiotic Strain from Infant fecal Flora

Golnoush Madani[1,2], Maryam Mirlohi[3*], Sabiheh Soleimanain-Zad[4], Parham Hosseini[5], Mina Babashahi[6]

[1] Food Security Research Center and Department of Food Science & Technology, School of Nutrition & Food Science Isfahan University of Medical Sciences, Isfahan, Iran

[2] Sir John Walsh Research Institute, University of Otago, Dunedin 9054, New Zealand

[3] Food Security Research Center and Department of Food Science & Technology, School of Nutrition & Food Science Isfahan University of Medical Sciences, Isfahan, Iran

[4] Food Sciences and Technology Department, Faculty of Agriculture, Isfahan University of Technology, Isfahan, Iran

[5] Department of Plant Pathology, Faculty of Agriculture, Isfahan University of Technology, Isfahan, Iran

[6] Community Nutrition Department, National Nutrition & Food Technology Research Institute, Faculty of Nutrition & Food Technology, Student Research Committee, Shahid Beheshti University of Medical Sciences, Tehran, Iran

*Correspondence should be addressed to Maryam Mirlohi, Food Security Research Center and Department of Food Science & Technology, School of Nutrition & Food Sciences Isfahan University of Medical Sciences, Isfahan, Iran; Tel: +983137922783; Fax: +983136682509; Email: m_mirlohi@hlth.mui.ac.ir.

ABSTRACT

Lactobacillus plantarum A7 was previously considered in some *in vivo* studies as a beneficial microorganism. This study deals with characterization of this strain based on general *in vitro* tests and its clear identification through molecular technique. A subtractive screening process was carried out on 26 *lactobacillus* strains. Based on tolerance to acid and bile and antibacterial activity against some pathogens, *L. plantarum* A7 ranked as one of the designated strains and further examined by some tests for probiotic properties like cell surface hydrophobicity, exopolysaccharide (EPS) production and adhesion to glass slides. In addition, antibiotic resistance and hemolytic activity were assessed for safety aspects. Molecular identification of this strain was performed by polymerase chain reaction (PCR), using primers 16 and 23-10c for genus level and planF and pREV for species. *L.* A7 revealed tolerance to acidic and bile containing environment against tested pathogen. Regarding the cell surface properties, this strain was compatible to commercial probiotic strains posing 53.6^{a}±4 and 72.07% ± 8.79 cell surface hydrophobicity towards toluene and adhesion to glass slid, respectively. This strain meets natural lactobacilli resistance to Vancomycin and lacks transferable resistant to Erythromycin and Cloramphenicol. In order to sequencing the PCR product, it was deposited in the GenBank database as *L. plantarum* KC355240. The results of this study could be considered as an accomplishing information to what was documented before on this strain with regard to its probiotic potential and it could be recommended to researchers, national and international organizations with high margin of safety.

Key words: Lactobacillus plantarum, Probiotic strains, Molecular characterization.

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1. INTRODUCTION

Probiotics are defined as live microrganisms which after administration, exhibited healthy profits on the host (1). The most studied strains of probiotics are from *Lactobacillus* and *Bifidobacterium* genera (1-4). About 60 species of the genus *Lactobacillus* have been recognized and are characterized by many metabolic activities that enable them to colonize various ecological niches like mammalian intestinal tracts (1-5). Characterization of every microbial strain as a Probiotic strain has been clearly defined by international organizations like the FDA (Food and Drug Administration, USA); belonging to nonpathogenic genera as well as being identified by accurate method is very important. In addition, in *in vitro* condition, microorganisms must impose defined properties including tolerance to acid and

bile, colonization properties, and antagonistic properties against pathogenic microorganisms. Demonstration of special antibiotic resistance pattern also is considered as an important trait for probiotics (6). During the last decades, molecular identification methods have been applied extensively for genotyping of new isolates. At the species level, there are several reports on specific PCR identification systems for lactobacilli, mainly based on the 16S rRNA or 23S rRNA or the sequence of the 16S-23S rRNA intergenic spacer region (ISR) (7). However, sometimes the rRNA genes do not allow to identification of closely related species. For example, the species *Lactobacillus plantarum*, *L. pentosus* and *L. paraplantarum* are genotypically closely related and show highly similar phenotypes while they can be rapidly distinguished using PCR amplification, targeting the ISR or housekeeping genes such as the *recA* gene (1, 4, 7). *RecA* is a small protein implicated in homologous DNA recombination, SOS induction and DNA damage-induced mutagenesis. Thanks to its fundamental role, the *recA* gene is ubiquitous, and its gene product has been proposed as a widely phylogenetic marker for distantly related species (7). In recent years, *L. plantarum* A7 was severally used in *invitro* and *invivo* studies by researchers working in different fields in Iran, particularly in the Isfahan University of Medical Sciences. However, this strain was not internationally identified and little information regarding the general probiotic properties of the given strain is documented in the literature. The objective of this study was to characterize *L. plantarum* A7 as a native potential probiotic. Precise molecular identification of this strain is focused by its registration in an international database.

2. MATERIALS AND METHODS

2.1. Materials and reagents

Microbial culture media, including de-Man- Rogosa Shape (MRS), Brain Heart Infusion (BHI) agar were from Merck (Germany). All solutions and chemicals were of analytical grade; hydrochloric acid, sodium chloride, di-hydrogen phosphate, ethylene diamine tetra acetic acid (EDTA), hexadecane, toluene, xylene, ethanol and tris were from Merck (Germany). Oxgal, Cetyl trimethyl ammonium bromide (CTAB), polyvinylpyrrolidone (PVP), β-mercaptoethanol, chloroform, isoamyl alcohol and isopropanol were provided from Sigma (USA). Antibiotic discs were purchased from Padtan-TEB (Iran). Taq DNA Polymerase (50 U.µL-1), deoxynucleotide triphosphates (dNTPs), PCR buffer (10X), Tris-borate-EDTA (TBE) Buffer (10X), ethidium bromide and Agarose were purchased from CinnaGen (Iran). DNA molecular weight marker III and 100-bp DNA ladder were provided from Sigma (USA). Used primers sequences were produced by Metabion International AG, Deutschland.

2.2. Apparatus

Co_2 incubator (Memmert, Germany), centrifuge (Ependorph, USA), Epi- fluorescence Microscope (Nikon, Japan), gel electrophoresis system and gel document (PayaPazhoohesh, Iran) and Techne Techgene Thermal Cycler were used to perform all the experiments in this study.

2.3. Microbial strains

Twenty-six *lactobacillus* strains which were previously isolated from infants' fecal flora, *L. rhamnosus* 519 a cheese isolated lactobacilli provided from the microbial collection in food microbiology laboratory at Isfahan University of Technology were tested for *in vitro* probiotic properties. *L. rhamnosus* GG was isolated from a pharmaceutical product (Culturelle, USA) and *L. acidophilus* Lad both were used as control to evaluation of hydrophobic properties. *L. plantarum* (ATCC: 13643) used as positive control in molecular identification. For antagonistic properties against harmful bacteria, pathogenic strains including *Escherichia coli* PTCC 1270, *Salmonella choleraesuis* subsp, *Cholerasuis serotypetyphimurium* PTCC1622, *Shigella dysentery* PTCC1188, clinical isolates of *Listeria monocytogenes* RITCC 1293 and *Escherichia coli* RITCC1293, clinical isolates of *Klebsiella pneumonia* and *Serratiamarcessense* PTCC1184 were obtained from microbial collection of Isfahan University of Medical Sciences.

2.4. Acid and bile tolerance test

The ability of the 26 human isolated lactobacilli to grow in the presence of bile was determined according to the method of Chou and Weimer with some modifications (8). Fresh culture of each isolated *lactobacillus* strain was individually inoculated in to MRS broth (1%), which was previously acidified to 3.5, 4, 4.5 and 5 (pH) with HCL (8N). In addition, bile resistant of the strains was assessed with the ability to grow in MRS medium containing 0.3 and 0.5 % oxgall.

2.5. Evaluation of antagonistic properties against pathogenic bacteria

Cell free culture supernatant (CFCS) of the examined strain was tested against pathogenic microorganisms using well diffusion method (3). Antibiotic resistance pattern was evaluated based on previous literature (9).

2.6. Total genomic DNA extraction

DNA was extracted as the following method: cells from successive overnight cultures were collected by centrifugation for 10 min at 4000g. The cells were treated with 800µ lysis buffer (5% CTAB + 1.4 M NaCl + 100mM Tris pH 8 + 20mM EDTA pH 8 + 2% PVP) and 8 µ β-mercaptoethanol for 30 min in 65ºC (Tubes were inverted during incubation every five minutes.). The samples then were incubated in 40º C for about 10 min. After addition of 1/2 volume chloroform/isoamyl alcohol (24: 1, v/v) and centrifugation for 15 min at 20817 g, the top layer was transferred into the new labeled tube and the last step was

repeated. The upper phase was emulsified with 2/3 volume of cold isopropanol, mixed by inverting tubes 10 times and centrifuged for 15 min at 18000g at 4º C. The supernatant was discarded and the pellet was washed in 200 µL 70% ethanol and centrifuged for 5 min at 18000 g at 4º C. The DNA was then precipitated, dried and suspended in 20 µL sterile water. In order to evaluate DNA concentration, an aliquot of the sample was run in an agarose gel electrophoresis with ethidium bromide. The fluorescence was then compared with amounts of standard DNA (DNA Molecular Weight Marker III).

2.7. DNA amplification conditions for Genus- and species-specific PCR

PCR primers (Table 1) were applied (16 and 23-10c) and (planF and pREV) and were purchased at Metabion International AG, Deutschland. For amplification of the 16S/23S SRs, a primer pair 16/23-10c was used and an almost 700 bp amplicon in size was expected for the genus *Lactobacillus*.

Table 1. Oligonucleotide sequences of the primers used in this study

Primer	Oligonucleotide sequence (5'- 3')	Length (bp)	%GC content	OD	Location
16	GCTGATCACCTCCTTTC	17	53	3	16S rRNA
23-10c	CCTTTCCCTCACGGTACTG	19	58	3	23S rRNA
planF	CCGTTTATGCGGAACACCTA	20	50	3	RecA
pREV	TCGGGATTACCAAACATCAC	20	45	3	RecA

Amplification was performed in a Techne Techgene Thermal Cycler. The reaction mixture (25 µl) contained 8 pmol of each primer, 0.2 mM of each deoxy-ribonucleotide triphosphate, 1U PCR buffer without $MgCl_2$, 1mM $MgCl_2$, 20 ng of bacterial DNA and 1U of Taq DNA Polymerase. Temperature cycling conditions for PCR were as follows:
An initial denaturation of 94°C for 3 min, followed by 30 cycles of 30s at 94°C, 30s at 57°C, and 30s at 72°C, and terminating with a 5-min final incubation at 72°C. 10 µl of the amplified PCR products were subjected to electrophoresis in 1.5 % agarose gels in TBE buffer pH=8.0 (44.5 mMTris-borate, 1 mM EDTA). A 100-bp DNA ladder was used as a reference. Gels were stained with ethidium bromide and visualized under UV light (7). The mixture of PCR reaction and amplification program for protein encoding *recA* gene were the same as those mentioned above, except that an annealing temperature of 56 was used instead of 57. The amplicons expected size was about 318 bp. The PCR products determined in this reaction were sequenced by a Macrogen sequencing company-Korea and have been deposited in the GenBank database.

2.8. Cell surface properties

In the present study cell surface property of probiotic candidates was assessed using three methods including cell surface hydrophobicity, EPS production ability and adhesion to glass slides were applied as determinant of the cell surface properties. Thee strains including LA7, *L. rhamnosus* 519 and *L. rhamnosus* GG underwent cell surface hydrophobicity examination and also compared for production of EPS. Bacterial surface hydrophobicity was determined by the cell adherence to hydrocarbons assay (10). For this test, active cultures of bacteria were prepared in MRS broth (18 hours), bacterial cells were provided by centrifugation (4500 g, 4 °C for 5 min). After washing by phosphate buffer, the sediment was re-suspended in phosphate buffer (pH = 7.2) to an optical density (OD 600) of 0.6–0.7. A test hydrocarbon (1 ml) (Hexadecane, Toluene and Xylene) was added to test tubes containing 3 ml of washed cells. The mixtures were blended on a vortex mixer for 90 s. The tubes were left to stand for 15 min for separation of the two phases and the OD of the aqueous phase was measured. Hydrophobicity was calculated from three replicates as the percentage decrease in the optical density of the original bacterial suspension due to cells partitioning into a hydrocarbon layer. The percent of hydrophobicity was calculated using the following equation:

$$\%\text{hydrophobicity} = (OD_{600} \text{ before mixing} - OD_{600} \text{ after mixing}) \times 100 / OD_{600} \text{ before mixing}$$

For the determination of EPS production ability, polysaccharide sediment method by ethanol was adapted from Degeest (11). Active bacterial culture in MRS broth was prepared (pH = 6.2). Five ml bacterial culture was centrifuged (g 11000, 5 min at 25 ° C) and the supernatant phase transferred to a sterile falcon. Equivalent to the volume of each supernatant, pure ethanol was added and was kept for 24 h at 4 ° C and was centrifuged (2000 g, 20 min at 25 ° C). The sediment was washed twice by 2 ml sterile distilled water and finally dried at 55 ° C for 24 hours. Then, falcon re-weighed. After deposition exo-polysaccharide, falcon weight gain in their internal level indicated the exo-polysaccharide production in terms of mg per 5 ml of culture, which was reported in 1 ml of medium cultures after conversion value. In adhesion test, stickiness to glass slide was examined using LA7 strain and commercial probiotic *L. acidophilus* Lad. An active culture prepared from two strains in MRS broth (pH = 6.2). Four ml of the culture was centrifuged (4500 g, for 5 min at 20 ° C). Bacterial deposition obtained by sterile phosphate buffer (pH = 7) and then washed with the same buffer was diluted to constant concentration of both strains provided (OD_{260} = 2.5). One ml of this medium was added to cylindrical glass container that each container was containing 100 ml of sterile buffer so that the turbidity was provided equal to 0.02 ± 0.006. Then a sterile glass slides were placed in each container and containers were incubated at 37 ° C for 4 hours. After this period, the slides were removed from the containers. Each slide was

immersed in a sterile plate containing 15 ml of sterile buffer for 10 s and was completely dried at room temperature. Then, the cells on the slide were stabilized by the gentle flame and were stained by Acridin Orange dye. Slide attached cells were counted under an epifluorescent microscope in 10 different visual field. The experiment was repeated 3 times and the results of LA7 strains were compared with the results of commercial strain (12).

2.9. Antibiotic resistant

Standard diffusion disc method was fallowed using MRS Medium and 21 different antibiotic discs; the average of 3 reading was reported as sensitive (8-12mm), relatively sensitive (1-8mm) and resistant (<1mm) (9).

2.10. Hemolytic activity

For testing hemolytic activity, fresh isolated strain was streaked on BHI medium containing 5% (w/v) sheep blood and incubated for 48h at 30°C.

2.11. Statistical methods

Experiments were replicated at least three times. Means and standard deviations were calculated using SPSS software (SPSS Inc., Chicago, IL, USA). The differences were considered significant when $P < 0.05$.

3. RESULTS AND DISCUSSION

3.1. The results of probiotic properties examinations

The results of acid and bile resistant tests showed that among the 26 tested strains, 8 strains less affected by the tested conditions, from which *L.rhamnosus* Sh4, *L. rhamnosus* 513 and two *L. plantarum* strains revealed outstanding tolerance to all used acid and bile conditions as they properly grew before 24 hours in MRS containing a different concentration of acid and bile (Table 2). The results of antagonist activity against pathogenic strains for three of the four tolerant strains identified in the previous test showed that all the pathogenic bacteria were more or less inhibited by the CFCS of the tested strains. However, after neutralization, only the CFCS obtained by *L. rhamnosus* 513 demonstrated antibacterial activity in a way that *L. monocytogenesis* and *B. cereus* inhibited by the given supernatant. In addition, *Listeria monocytogenes* and *Shigella dysentery* were shown to be the least and most sensitive strains to LA7 in two replicates of three individual tests.

Table 2. Evaluation of acid and bile tolerance of examined *lactobacillus* strains based on ability to growth in MRS broth containing mineral acid and oxgall

Lactobacillus strains	Ability to growth in MRS medium with different pH			Ability to growth in Oxgall containing medium (w/v)	
	pH: 4.5	**pH: 4**	**pH: 3.5**	**0.3%**	**0.5%**
L. acidophilus					
H13	+	W	-	-	-
H110	+	W	-	+	W
H1102	+	W	-	+	W
H19	+	+	+	W	-
H26	+	+	+	+	W
H22	-	-	-	W	-
H28	W	-	-	+	-
H24	+	+	+	W	-
SH4	+	+	+	+	+
SH41	+	-	-	W	-
SH5	+	-	-	W	-
SH51	+	-	-	W	-
L. fermentum					
H27	+	+	W	+	+
L. rhamnosus					
13	+	+	-	+	W
3	+	+	-	+	+
111	+	W	-	+	W
15	+	W	-	+	W
513	+	+	+	+	+
G3	W	W	-	+	-
G4	W	-	-	+	+
L. casei					
14	+	-	-	+	-
17	+	+	W	+	+
18	+	W	W	+	-
L. plantarum					
A7	+	+	+	+	+
A71	+	+	+	+	+
A8	+	+	W	+	+

Positive (+), Negative (-) and weak (W) reactions were regarded as growth before 24 hours , lack of growth before 48 hours, and growth after 24 hours but before 48 hours, respectively.

In Table 3 the result of cell surface hydrophobicity and EPS production of 3 tested strains have depicted. Both LA7 and L. 519 presented higher affinity to toluene than L.GG as a commercial control strain. However, using hexadecane as an organic material, there was no significant difference (P>0.05) in the cell surface hydrophobicity among the three tested strains.

Table 3. Cell surface hydrophobicity % of three tested *lactobacillus* strains*

	Toluene	Hexadecane
L. plantarum A7	53.6 ± 4^{a}	33.3 ± 5.8^{a}
L. rhamnosus 519	35.7 ± 6.4^{a}	35.7 ± 3.2^{a}
L. GG	13.8 ± 7.5^{b**}	31.5 ± 8.5^{a}

*Each value is expressed as mean ± SD in triplicate experiments.
**Values with different alphabets are significantly different at p<0.05.

Evaluation of EPS production ability showed that examined strains produced 27.5 to 50 mg polysaccharide. With no significant difference (P>0.05) between LA7 and LGG they produced 30±2.4 mg L^{-1} and 37±5.1 mg L^{-1} EPS respectively. In addition, *L. rhamnosus* 519, a cheese isolated lactobacillus strain, deposited 47±2.5 mg L^{-1} EPS which was significantly higher than the other two strains. In the adhesion test, attached cell counting was directly enumerated in 10 microscope magnification (Mean ± SD). Comparison of LA7 with a commercial probiotic *L. acidophilus* Lad to glass slide were accounted as 72.07% ± 8.79 and 46.9% ± 15.9, respectively. This difference was

not statistically significant (data was not shown). Regarding antibiotic resistance, LA7 was sensitive to the most cell wall synthesis inhibitors and antibiotics, especially, Co-amoxyclave C 30 and Ceftriaxone C 30 but resistant to Vancomycin C 30. LA7 was resistant to the most of the nucleic acid synthesis inhibitors with the exception of Rifampicin and Nitrofurantoin. In addition, it was shown that this strain was highly sensitive to Erythromycin, Ampicilin, and Penicillin and resistant to Ciprofloxacin. LA7 was able to grow on Blood Agar Medium, but characterized as Gamma hemolytic bacteria.

3.2. Molecular identification of LA7

Using the 16S-23S ISR and its flanking 23SrRNA analysis of the tested strain was identified as a lactobacilli (Figure 1-A). Using *recA* gene sequencing comparison (Figure 1-B), the size of the tested strain amplicon was 318 bp which is belonged to *L. plantarum*. This PCR product has deposited in the GenBank database.

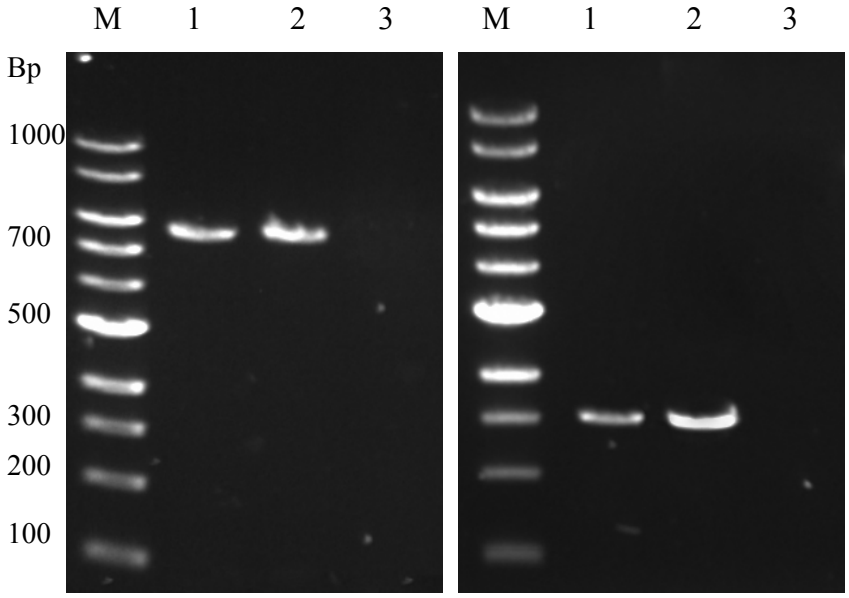

Figure 1. Agarose gel (1.5 %) analysis of PCR amplification. A: PCR products using 16 and 23-10c primers for genus identification. B: PCR products using *recA* species specific primers. In both figures, first lane (M) is 100 bp ladder, followed by 1): positive control (reference strain of *L. plantarum* (ATCC: 13643)), 2): our isolate and 3): negative control, respectively

Every potential probiotic strain should be extensively studied and reviewed before recommendation to public. Food safety organizations, like the FDA in the United State of America and European Food Safety Authority (EFSA) in the European Union do this task by collection and scientifically evaluation of verity of studies on a particular strain. Generally, all the data in connection to a candidate strain should be properly documented in the literature. *L. plantarum*A7 is one of the solely reported isolates form a safe, human source in Iran. Nevertheless, due to lack of information, *L. plantaum*A7 responds to general *in vitro* tests with lack of its molecular recognition. This study was conducted to provide more detailed figures on this strain to present better view of that for national agencies. Moreover, the precise and exact identification of this strain would bring the possibility of using this strain in more studies with a larger margin of safety. This study began by subtractive screening of some lactobacillus strains from fecal flora through acid and bile resistance following antagonistic activity against pathogens which resulted in characterization of *L. rhamnosus* 513 and *L. plantarum* A7 as the candidates for further investigation. With regard to anti pathogenic effect, inhibition of pathogens by the cell free supernatant of lactic acid bacteria has broadly reported before justifying by production of organic acids, diacetyl, Acetoine, 2,3 butandiol , phenyl lactic acid and hydroxyl phenyl lactic acid (13-15). Instead, efficiency of the neutralized cell free supernatant (CFS) is not a common characteristic for LAB and only some strains are able to produce bacteroinces. For L.513 production of specific antimicrobial peptides or lysozyme might be involved in, because its neutralized CFS affected the gram-positive indicators. Nonetheless, there was a great limitation regarding *L. rhamnosus* 513 usage as it was so sensitive to aerobic condition. Makras et al reported that probiotic lactobacilli are able to produce more than 150mm lactic acid in their specific culture (MRS) as the particular inhibitory compound against *Salmonella typhi*. However, they introduced an unknown, non-proteinases compound which was being active in pH=4.5 and in the presence of 125mmol lactic acid which contributes antibacterial properties of *L. plantarum* ACA-DC 287 (13). Similarly, Nikopavalan et al for *L. plantarum* E78 described a monolacton compound with bacterial inhibition effect (15). With regard to cell surface properties, LA7 showed compatible characteristics to commercial probiotics. A number of studies support this assumption that cell surface properties could be indicative for their beneficial effects in the gut (16-18). Drakseler et al indicted that strains, resented 37 to 70% cell surface hydrophobicity, were more prone to accumulate and were considered as superior probiotic candidates (16). Varied affinity to different

hydrocarbons for each strain which was observed in this study, was previously reported by Mishra et al (18). Some studies reported that lactobacillus strains are able to produce EPS by an amount of 40- 300 mg L^{-1}; while EPS produced by the tested strains in this study were less than the mentioned range. The ability to EPS production for lactobacilli is of importance for gut colonization and for being used in the food industry. Regarding anti-bigram profile, LA7 meets the natural antibiotic resistance of lactobacilli to vancomycin and lacks acquisitive and transmissible resistant to Erythromycin and Chloramphenicol. In addition, it characterized as gamma hemolytic while place as a safe strain that was more confirmed. This contributed the previous assumption that this strain is quite safe. Because, in one study, blood, spleen and liver samples of mice, upon ingestion of LA7 were investigated for translocation and all the translocation tests were negative (2). Despite the absence of any pathogenicity of this strain, it was not clearly identified by molecular techniques and could not be considered as a probiotic candidate. *L. plantarum* species is very closely related to two other strains, including *L. paraplantarum* and *L. pentosus*; so that to guarantee species identity, the *recA* gene sequences was used behind ISR region. Among the *lactobacillus* species, *L. plantarum* is known as the most adaptable *Lactobacillus* species inhabiting a wide verity of environmental niches. Its larger genome, higher ability in metabolizing different carbon sources and growth ability were stated to be responsible for its adaptability (3). There are several different reports in the literatures on the *L. plantarum* strains colonization and population in the human intestinal flora (19). From which, some revealed that *L. plantarum* is a dominant *lactobacillus* species in Infants and children (19-21). This helps to introduce a potential probiotic strain belonging to *L. plantarum* species. The beneficial effects of a special strain has been extensively revealed by the several pieces of research and its application as a potential probiotic strain yet to meet the required criteria of probiocity. the given strain need to be clearly identified as an inhabitant of human gut microbiotia, L.A7 as *L. plantarum* strain that was registered as KC355240, characterized and being used as a potential probiotic with native origin (22).

4. CONCLUSION

Overall, based on the results obtained in this study, *in vitro* probiotic properties featured by LA7 compile with the criteria accepted for probiotic microorganism. This strain showed compatible resistance to acid and bile and adhesion properties supporting its functional characteristic reported by several *in vivo* examinations. Exact molecular identification of LA7 and its antibiotic resistance profile revealed in this study, provide the safety requirements of food grade microorganisms and facilitate its future application in research studies or therapeutic treatments.

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AUTHORS CONTRIBUTION

All authors discussed the results and contributed to the final manuscript. G. Madani, M. Mirlohi, carried out the experiments, prepared the draft and wrote the manicurist. S. Soleimanian-Zad supervised the project, P. Hosseini made a substantial contribution to the molecular experiments and M. Babashahi contributed to the concept, experiments and final revision of the manuscript.

CONFLICT OF INTEREST

The authors declared no potential conflicts of interests with respect to the authorship and/or publication of this paper.

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