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Technological properties of candidate probiotic *Lactobacillus plantarum* strains isolated from Iranian traditional Lighvan cheese

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ABSTRACT

Lighvan cheese is one of the mostly used popular types of traditional Iranian dairy product. This type of cheese is made basically from raw milk of sheep, accompanied by 20-30% goat milk without starter culture. The pleasant smell and taste of this cheese indicates that different useful types of native microorganisms are activating in the production and ripening period of this cheese. Thus, these cheeses are good sources of different beneficial microbes which could be used in starter cultures for the production of fermented dairy products. Therefore, the aim of this study was to evaluate the technologically relevant characteristics of seven strains isolated from traditional Lighvan cheese in order to determine their potential in the development of new starter cultures. To this end, the proteolytic and lipolytic activities of the strains in culture media were firstly assessed. Then, these activities were evaluated through PCR technique. Finally, all test strains were evaluated for their viability in simulated gastric condition. The results showed that all isolates at 30°C and all strains except *L. plantarum*5 at 37°C were attributed proteolytic activities. All strains except *L. plantarum* isolates 1 and 7 showed lipolytic activities at 30°C and at 37°C only strains of *L. plantarum* isolates 1,2,3,4 showed lipolytic activities. It was also identified that from 7 test isolates, 3 isolates of *L. plantarum*, isolate 1, 2, 3 could potentially be represented as probiotic according to their resistance to the available conditions in upper gastrointestinal transit. Hence, the result of this study confirmed that traditional dairy product with the indigenous probiotic flora could be a good candidate for isolation and development of new starter culture.

Keywords: *Lactobacillus plantarum*; Technological properties; probiotic potential; cheese

INTRODUCTION

There is a continuing need to improve existing cultures or to select new strains to be used as starters in specific fermentation processes and in development of new products. In dairy fermentations, a stable and predictable rate of acid production is required; so the ability to metabolize lactose by the starter strains is one of the most important properties. Other important metabolic properties of starter cultures are proteolytic activity, salt tolerance, biogenic amines production, bacteriocin production, susceptibility to bacteriophage attack, and extracellular polysaccharide production. It is well known that many of these biotechnologically important properties of lactic acid bacteria, mainly lactose metabolism and proteolytic activity, are associated with plasmids; so their presence in starters also needs to be investigated [1].

Lactobacilli are among the most important lactic acid bacteria (LAB) used in the food production and are gaining increasing attention in the area of probiotics [2, 3]. Within the genus *Lactobacillus*, *Lactobacillus plantarum* is a member of the facultatively heterofermentative group of lactobacilli. It is a heterogeneous and versatile species that is encountered in a variety of environmental niches, including dairy, meat, fish, and many vegetables or plant

fermentations. *L. plantarum* strains have also been found in many cheese varieties. Moreover, strains of *L. plantarum* have confirmed ability to survive in gastric transit conditions and colonize the intestinal tract of humans and other mammals [4-6]. Various specific therapeutic or prophylactic properties have been associated with *L. plantarum*, such as reduced incidence of diarrhea in daycare centers, reduced pain and constipation associated with irritable bowel syndrome, reduced bloating, flatulence, ability to displace enteropathogens from Caco-2 cells, and capacity to exert positive effect on immunity in HIV⁺ children [7-9].

Lighvan cheese is one of the mostly-used popular types of traditional Iranian dairy product which is produced in southeast of Tabriz, Iran. This type of cheese is made basically from raw milk of sheep, accompanied by 20-30% goat milk without starter culture. It has a mild taste and shows acidic characteristics. Nowadays, Food and Drug Administration (FDA) persuades the manufacturers to use pasteurized milk in the cheese production. However, manufacturers believe that the specific pleasant smell and taste of Lighvan cheese is the result of use of raw milk and that is due to the activities of proteolytic and lipolytic enzymes, produced by indigenous microflora of raw milk. The pleasant smell and taste of this cheese indicates that different useful types of native microorganisms are activating in the production and ripening period of this cheese [10]. Thus, these cheeses can be good sources of indigenous probiotic candidates and edible germs which could be used in the production of starter cultures. Therefore, the aim of this study was to evaluate the technologically relevant characteristics of seven strains of *L. Plantarum* isolated from traditional Lighvan cheese, in order to determine their potential in the development of new starter cultures.

MATERIALS AND METHODS

The isolates of *Lactobacillus plantarum*

Seven *Lactobacillus plantarum* isolates were used in this study. They were isolated from traditional Lighvan cheese and were confirmed by Biochemical characteristics and amplification of 16S rDNA (Data not shown).

proteolytic activity

In order to determine the proteolytic activities of strains, the identified isolates were cultivated on reconstructed agar plates (10%) containing skim milk medium and were heated at 30°C for 18-20 h. Colonies having a transparent ring around them were considered as strains indicating proteolytic activity [11].

To confirm the proteolytic activities of isolates and compare them with culture method, total genomic DNA was extracted from broth cultures of the strains according to the method reported by Marmur, 1961 [12]. Amplification of the 16s rDNA was carried out using the primer pair reported previously Sanchez et al., 2005 [13]. P6(S):5'-CAACACGGCATGCATGTTGC-3' and P7(A): 5'-CTGGCGTTCCCACCATTCA-3'. PCR amplification was performed using master mix (Ampliqon, Herlev, Denmark), 0.4 μM primer, 40 ng genomic DNA, in 25 μl final volume. The cycling program was as follow: denaturation at 94°C for 4 min, 32 cycle of: 94°C, 50 sec, 59°C, 50 sec, 72°C for 60 sec and a final extension was performed 5 min in 72°C. The PCR products were run in 1% agarose gel and stained by DNA stain SYBR green (Rima Sight).

lipolytic activity

To determine the lipolytic activities in strains, identified isolates were cultivated on medium plate containing Nutrient agar and 1% (v/v) & cream of milk (38% fat) and incubated at 37°C for 72 h. Colonies having a transparent ring around them were considered as strains indicating lipolytic activity [14-15].

To confirm the lipolytic activities of strains and compare them with culture method, PCR was carried out by specific primers that was designed by NCBI primer Blast software [16]; (LPLF: 5'-GATGCCAACAATTAATTCG-3' and LPLR: 5'-CTAAATTAACGCGGCCGC-). The cycling program for PCR was as follow: denaturation at 94°C for 4 min, 32 cycle of: 94°C, 50 sec, 55°C, 50 sec, 72°C for 60 sec and a final extension was performed 5 min in 72°C.

Tolerance to simulated gastric Juice

To this end, the isolates were separately cultured in 10ml MRS broth medium and incubated at 37°C for 24 h under anaerobic conditions. Then 5 ml of the 24h media were separately poured in 2 tubes and were centrifuged at 4000 rpm for 15 m. Then the liquid was removed from the surface and 2 ml gastric juice (HCL 0.08 mol containing 2% NaCl, pH = 1.55 without pepsin) was added to the sediment in tube 1 and 2 ml sterilized physiological serum was added to sediment in tube 2. The samples were incubated at 37°C and after 2 h, the resulted mixtures were centrifuged at 5000 rpm for 30 m. The liquid was removed from the surface again and 2 mL of physiological serum was added to the sediment and solved. The serial dilution was made for the suspension. These dilutions were cultured on MRS agar and incubated anaerobically at 37°C for 48 h. Two days later, the number of colonies and survived bacteria in acidic conditions were investigated and assessed [17-18].

Bile resistance

The isolates were separately cultured in MRS broth medium for 24 h. Then 1 ml of the overnight media was added to the MRS broth medium containing 0.3% bile salt and 1 ml was added to the MRS broth medium without bile salt. The samples were incubated at 37°C for 6 h. Starting incubation, light absorption of each sample was measured per hour by spectrophotometer at a wavelength of 500 nm (Data not shown). From beginning of time, 1ml of mentioned culture media was diluted every 2 hours. Then 0.1ml of the three latter dilutions, were separately cultured in MRS agar moderately and plates were incubated at 37°C for 48h. The results were then investigated and assessed [19-23].

RESULTS AND DISCUSSION

The results of this investigation show differences in characteristics among strains and confirm the necessity reported by other authors [24-26]. of assessing its potential before selecting a strain as a starter culture for food fermentations [27].

Table 1: Average number of isolates' cells survived in media with and without bile in 6 h of incubation

Isolates	Bile	Time (hour)			
		0	2	4	6
<i>L. plantarum1</i>	+	51×10^7	38×10^7	38×10^7	14×10^7
	-	48×10^7	61×10^7	34×10^7	64×10^7
<i>L. plantarum2</i>	+	253×10^7	23×10^7	32×10^7	42×10^7
	-	263×10^7	196×10^7	84×10^7	181×10^7
<i>L. plantarum3</i>	+	11×10^7	111×10^7	186×10^7	92×10^7
	-	64×10^7	78×10^7	35×10^7	22×10^7
<i>L. plantarum4</i>	+	6×10^7	361×10^7	156×10^7	201×10^7
	-	3×10^7	2×10^7	7×10^7	6×10^7
<i>L. plantarum5</i>	+	175×10^7	73×10^7	88×10^7	163×10^7
	-	29×10^7	178×10^7	156×10^7	73×10^7
<i>L. plantarum6</i>	+	29×10^7	3×10^7	4×10^7	5×10^7
	-	78×10^7	63×10^7	84×10^7	194×10^7
<i>L. plantarum7</i>	+	232×10^7	81×10^7	63×10^7	93×10^7
	-	196×10^7	202×10^7	281×10^7	214×10^7

In order to display the tolerance of isolates to the present conditions in upper gastrointestinal transit, studied isolates' cells were exposed to simulated gastric juice in two stages. These cells were placed for 2 h (which is the time required for stomach content to be emptied nearly completely) in environment with pH 1.55 [28] and then for 6 h in bile environment (Bile) with 0.3%, w/v density. As observed in table 1, from examined strains, 3 strains of *L. plantarum1*, *L. Plantarum2* and *L. Plantarum5* show high survival rate in conditions with pH equal to human stomach condition. On the other hand, results in table 2 show that all test isolates except *L. plantarum6* show high survival rate in 2 beginning hours and even till 6 h in conditions similar to the condition of upper gastrointestinal transit. All results show that from 7 test isolates, 3 isolates of *L. plantarum1*, *L. Plantarum2* and *L. Plantarum5* can potentially be represented as probiotic microorganisms according to their resistance to the conditions prevalent in upper gastrointestinal transit.

Table 2: average number of isolates' cells survived in acidic (pH=1.55) and non-acidic (pH=7) conditions after 2 h of incubation

Isolates Medium	<i>L. plantarum1</i>	<i>L. plantarum2</i>	<i>L. plantarum3</i>	<i>L. plantarum4</i>	<i>L. plantarum5</i>	<i>L. plantarum6</i>	<i>L. plantarum7</i>
Non-Acidified	34×10^7	223×10^7	331×10^7	186×10^7	334×10^7	728×10^7	614×10^7
Acidified	73×10^6	43×10^6	-	-	35×10^6	-	2×10^6

Table 3: proteolytic activities of isolates after incubation at 30°C and 37°C for 72 h

Isolates Temperature	<i>L. plantarum1</i>	<i>L. plantarum2</i>	<i>L. plantarum3</i>	<i>L. plantarum4</i>	<i>L. plantarum5</i>	<i>L. plantarum6</i>	<i>L. plantarum7</i>
30 °C	+	+	+	+	+	+	+
37 °C	+	+	+	+	-	+	+

The results of assessment of proteolytic ability of test isolates have been shown in table 3. For this assessment, the temperatures of 30°C and 37°C were used for 72 h. The results indicated that all isolates show proteolytic activities

at 30°C and so do all strains except *L. plantarum*5 at 37°C. Hebert and et al (2000) reported that in the lactobacillus strains they studied, some represented high proteolytic ability while others represented poor activity [1]. These strains were called fast coagulating and slow coagulating, respectively. Of course, some of the strains showed proteolytic activity when they were enriched with yeast extract or casein enzymatic hydrolysate. They also concluded that the proteolytic activity of Thermophilic lactobacilli strains studied, are not coded with plasmid[1]. Reinheimer and et al (1996) have proposed that proteolytic activities are related to the strains [29]. As it is observed in table 3, proteolytic activities of strains are related to the temperature and this can be observed in the results reported by Hebert and et al (2000) [1].

Table 4: Lipolytic activities of isolates after incubation at 30°C and 37°C for 72 h

Isolates	<i>L. plantarum</i> 1	<i>L. plantarum</i> 2	<i>L. plantarum</i> 3	<i>L. plantarum</i> 4	<i>L. plantarum</i> 5	<i>L. plantarum</i> 6	<i>L. plantarum</i> 7
Temperature							
30 °C	-	+	+	+	+	+	-
37 °C	+	+	+	+	-	-	-

In order to assess the lipolytic activities of strains, the temperatures of 30°C and 37°C were used for 72 h. The results were related to temperature and incubation time (table 4). All strains except *L. plantarum*1 and *L. Plantarum*7 isolates showed lipolytic activities at 30°C. And at 37°C only *L. plantarum*1, *L. Plantarum*2, *L. Plantarum*3 and *L. Plantarum*4 strains showed lipolytic activity. As it is observed in figure 1, isolates 2, 6 and 7 have the band of protease gene and isolate 6 had lipase gene band.

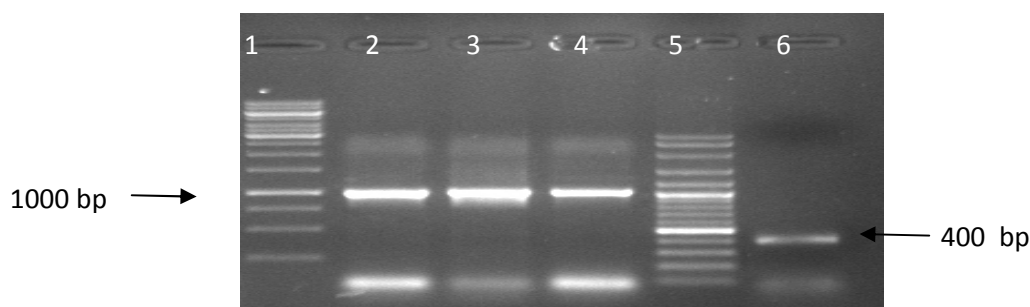


Figure 1. Agarose gel electrophoresis analysis for the protease and lipase genes in *Lactobacillus plantarum* strains

Lane 1, 1kb DNA Ladder (*fermentase*, SM0311); lane 2, *L. Plantarum*2; lanes 3, *L. Plantarum*6; lane 4, *L. Plantarum*7; Lane 5, 100 bpDNA Ladder (*Vivantis*, NL1407) and lane 6, *L. Plantarum*6. Presence of the 400 bp shows the lipase gene existence in the isolates and the 1000bp shows the protease gene existence in the isolates.

The results of this study showed that traditional dairy product with the indigenous probiotic flora could be a good candidate for isolation and development of new starter culture and the careful choice of natural isolates will be essential for successful development of new starters.

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