Isolation and study on the technological and probiotic characterization of Lactobacilli in traditional white Lighvan cheese

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Abstract:

BACKGROUND: Probiotics and especially Lactobacilli are among the most important components widely used in food technology. Isolation and characterization of indigenous probiotics should be performed in native populations. **OBJECTIVES:** In order to isolate lactobacillus spp. from Iranian traditional Lighvan cheese to be used as starters, 15 cheese samples were randomly selected and tested for technological and probiotics properties. **METHODS:** Lactobacilli were characterized using culture-based and biochemical techniques and results were verified using PCR analyses of the 16S rRNA region. The probiotic characteristics of these bacteria were studied by acid and bile tolerance. The susceptibility of different isolates against a number of important antibiotics was tested. The technological traits of LAB were assessed using measurement of acid production and analyzing salt tolerance. The isolates were also screened for proteolytic and lipolytic activity. **RESULTS:** Of the 12 samples containing Lactobacilli, 10 belonged to L. plantarum while 2 were close to L. bulgaricus spp. All isolates were tolerant to 3% NaCl and 9 tolerated 6-8% NaCl. Almost all isolates were lipase-positive at 37 °C and 30 °C. Nine Lactobacilli were shown to be protease-positive at 37°C. Two isolates were completely tolerant to pH 1.55, 4 had intermediate resistance, 4 were of weak resistance and 1 was sensitive. All but one isolate were tolerant to 0.03% bile acid. Some isolates were shown to be sensitive to erythromycin, ampicillin, tetracycline, amikacin and rifampicin. **CONCLUSIONS:** Results indicate that most of the isolated Lactobacilli can be used as industrial cheese starters and/or as native probiotics in production of functional foods.

Introduction

For many centuries, Lactobacilli have been incorporated in fermented milk, cheese, yogurt and ice cream. Lactic acid bacteria (LAB) have been successfully used to prevent antibiotic associated diarrhea and to treat acute infantile diarrhea, recurrent Clostridium difficile infection and other diarrheal illnesses (Masood, Qadir, Shirazi, & Khan, 2011). In the years to come, probiotics may be indicated for prevention and treatment of several other conditions. These therapeutic benefits have been attributed to the lowering of pH caused by introduction of LAB and production of other primary and secondary antimicrobial metabolites (Boirivant & Strober, 2007; Vanderpool, Yan, & Polk, 2008). Furthermore, probiotics enhance the immune status

of the host (Boirivant & Strober, 2007). The metabolites produced by fermentation are kept in the foods and result in growth inhibition of food spoilage, prevention of bacterial growth and detoxification of noxious compounds with plant origin (Daeschel, 1989). LAB produce various antimicrobial compounds, which can be classified as low-molecular-mass compounds such as hydrogen peroxide, carbon dioxide, diacetyl(2,3-butanedione), uncharacterized compounds, and high-molecular-mass compounds such as bacteriocin (Daeschel, 1989). These compounds can antagonize the growth of pathogenic bacteria in food. Most of the probiotic Lactobacilli in human foods are supplied in highly-concentrated forms containing more than 10^{10} cfu g^{-1}. These concentrates are usually freeze dried, spray dried or micro-encapsulated (Mattila-Sandholm, et al., 2002). Very recently, it has been recommended that if probiotics are isolated from native microbiomes, they are likely to exert more benefits in the natives (Barzegari & Saei, 2012). In line with our previous studies on isolation of Lactobacilli from traditional cheese types (Mirzaei & Homayouni, 2011; Mirzaei & Barzegari, 2012), in the current study we have focused on traditional cheese from Eastern Azerbaijan.

In this work, we aimed to characterize the strains of Lactobacillus spp. present in Iranian traditional Lighvan cheese as well as their technological properties and probiotic potential. Materials and methods:

Sample collection and isolation of Lactobacilli: Fifteen samples of traditional Lighvan cheese were randomly selected and tested for presence of Lactobacillus strains. For this purpose, 25 g of each homogenized sample was diluted in 225 mL of sodium citrate 2%. Then, 10 mL of the resulting dilution was transferred to 90 mL MRS broth and incubated at 37°C for 24 h. 0.1 mL of the resulting medium was cultured in the MRS agar and then incubated in an anaerobic condition at 37 °C for 48-72 h. Colonies were evaluated by catalase test and Gram staining and samples of rod-shaped, Gram-positive and catalase-negative bacteria were incubated in anaerobic condition in MRS broth at 37°C for 24 h to prepare stocks and extract DNA.

DNA extraction, PCR of 16S rRNA regions and Amplified Ribosomal DNA Restriction Analysis (ARDRA): For extraction of DNA, 1.5 mL of MRS broth was transferred to test tubes and centrifuged at 12000 rpm for 5 min. The supernatant was discarded and the pellets were used for DNA extraction. Liquid nitrogen was then used to break the bacterial cell walls and expose DNA. Finally, the extracts were transferred to microtubes using 1 mL of lysis buffer pH 8 (Tris-base 12.1 g, EDTA-Na2 7.44 g, PVP 2% 1g, NaCl 81.82 g, C-TAB 20 g and brought to 1000 mL with distilled water), homogenized by shaking and then incubated at 60°C for 1 h. The procedure was followed by centrifugation at 12000 rpm for 5-10 min; the supernatants were transferred to new 1.5 mL tubes and chloroform isoamyl alcohol was added in equal volumes. Tubes were shaken by hand 3-4 times slowly and were then centrifuged at 12000 rpm for 10 min. The transparent liquid on top was gleaned carefully using a sampler and transferred to the new tubes and cold isopropyl alcohol was added at equal volumes and shaken. Tubes were kept at -20°C for 30 min and were then centrifuged at 12000 rpm for 10 min. Supernatant was removed and tubes were left to dry at laboratory temperature. Before PCR analyses, 50 µL of distilled water was added to the tubes to rewet DNA.

PCR of 16S rRNA was performed using a thermocycler (S1000™ Thermal Cycler, BIO-RAD) at a reaction volume of 25 µL containing 0.5 µL of each of the forward and reverse primers, 50 ng (1 µl) DNA template, 12.5 µL Master mix and 10.5 µL of deionized H2O. Initial denaturation was performed at 94°C for 5 min, 32 cycles of 94 °C for 1 min, 57°C for 1 min, 72°C for 1.5 min and finally, one cycle at 72°C for 10 min. Electrophoresis gel was stained by CyberGreen. PCR forward (5´-AGAGTTTGATC-MTGGCTCAG-3´) and reverse (5´-TACCTTGT-TAGGACTTCACC-3´) primers were designed according to our previous study (Mirzaei & Barzegari, 2012). After gel electrophoresis (1% agarose in TAE 1X) in 85-100 mV for 1-1.5 h, gel was stained using CyberGreen, viewed and photographed under gel dock.

After amplification of the 16S rRNA regions, the fragments were exposed to the restriction enzyme TaqI. Digestion was carried out for 12 h in controlled temperature (3 µL of the PCR product, TaqI 1µL, buffer 2 µL and deionized water 14 µL). Digestion products were subjected to gel electrophoresis (1.5% agarose) and viewed by gel dock. The electrophoresis

patterns were compared to the results obtained using virtual digestion of 16S rRNA sequences retrieved from NCBI using gel dock software.

Biochemical tests: For characterization of the Lactobacilli, Gram staining and catalase test were carried out.

Production of acid: 3 mL of the Lactobacilli MRS culture was transferred to a 250 mL Erlenmeyer flask containing milk in sterile conditions. The cultures were incubated at 37°C. pH of the cultures was measured using pH meter (HORIBA Scientific, USA) in 0, 4, 8, 24, 48, 72 and 96 h.

Acid, bile and salt tolerance: Two mL of the MRS broth was transferred into a 100 mL Erlenmeyer flask containing sterile milk under sterile conditions. For each tested Lactobacillus, 4 Erlenmeyer flasks were used containing 3, 6, 8 and 10 g of NaCl. The Erlenmeyer flasks were incubated at 37°C. The pH was measured at 0, 4, 8, 24, 48 and 72 h.

For testing acid tolerance, the isolated and characterized Lactobacilli were cultured in 10 mL MRS broth and incubated for 24 h at 37°C under anaerobic conditions. 5 mL of the culture was transferred to 2 test tubes and centrifuged for 15 min at 4000 rpm. The supernatant was discarded and 2 mL of the gastric fluid (0.08 M HCl, 0.2% NaCl, pH 1.55) and 2 mL sterile physiologic serum were added. Samples were incubated at 37°C and after 2 h were centrifuged at 5000 rpm for 30 min. The supernatants were discarded, 2 mL of physiologic serum was added and test tubes were centrifuged at 4000 rpm for 15 min. The supernatant was discarded and 2 mL of physiologic serum was added to the precipitate. Serial dilutions were prepared from the resulting suspension and cultured on MRS agar. The cultures were incubated for 48 h at 37°C under anaerobic conditions and then, colonies were counted.

Sample Lactobacilli were cultured for 24 h at MRS broth. 1% of the culture was added to MRS broth containing 0.3% bile acids (Oxgall). MRS without bile acids was considered as control. The optical density of cultures was determined every 30 min using a spectrophotometer up to 6 h. Then, samples were incubated at 37°C for 24 h, after which optical density 600 of the cultures was measured again.

Protease and lipase activity: To determine if the isolated bacteria possess proteolytic activity, isolates were cultured onto fat-free plates containing milk and 10% agar. Plates were incubated in 30°C for 12-20 h. To confirm the experimental results, previously isolated DNAs were subjected to PCR analyses using forward (5´-CAACACGGCATGCATGTTGC-3´) and reverse (5´-CTGGCGTTCCCACCATTCA-3´) primers previously designed by Klijn et al. (Klijn, Weerkamp, & De Vos, 1995). L. fermentum ATCC was used as positive control throughout PCR.

To determine the lipase activity, the isolated Lactobacilli were cultured on nutritive agar 1% (v/v) and milk cream (38% fat) and incubated at 37°C for 72 h.

Antibiotic resistance: Disk diffusion agar method was used to determine the susceptibility of isolated Lactobacilli to 8 antibiotics, namely ampicillin (10 µg), amikacin (30 µg), erythromycin (15 µg), norfloxacine (10 µg), penicillin (10 UI), rifampicin (30 µg), streptomycin (10 µg) and tetracycline (30 µg). 0.5 McFarland suspensions of the bacteria was prepared and cultured on Mueller-Hilton agar. Antibiograms of each isolate were done using 8 antibiotic disks and the resulting plates were incubated in 30°C for 24 h.

Results

16s rRNA analysis: In figure 1, sharp bands with 1500 bp size correspond to the 16S rRNA genes of different Lactobacilli. Only 12 out of 15 samples were found to contain Lactobacilli. Isolated Lactobacilli were encoded as LB1 through 12 for further experiments.

For more accurate characterization of isolated Lactobacilli, ARDRA method along with bio-informatic analysis of the results using Gene Doc software was used. The digestion pattern of sequences obtained from NCBI indicated that 10 isolates belonged to L. plantarum while 2 corresponded to L. bulgaricus (figure 2).

Production of acid by isolates: Different isolates were incubated in milk at 37°C to assess the amount of acid production in order to characterize the technological potential of the Lactobacilli in fermentation processes. The results have been presented in figure 3.

Acid, bile and salt tolerance: The results from acid tolerance have been presented in Table 1.

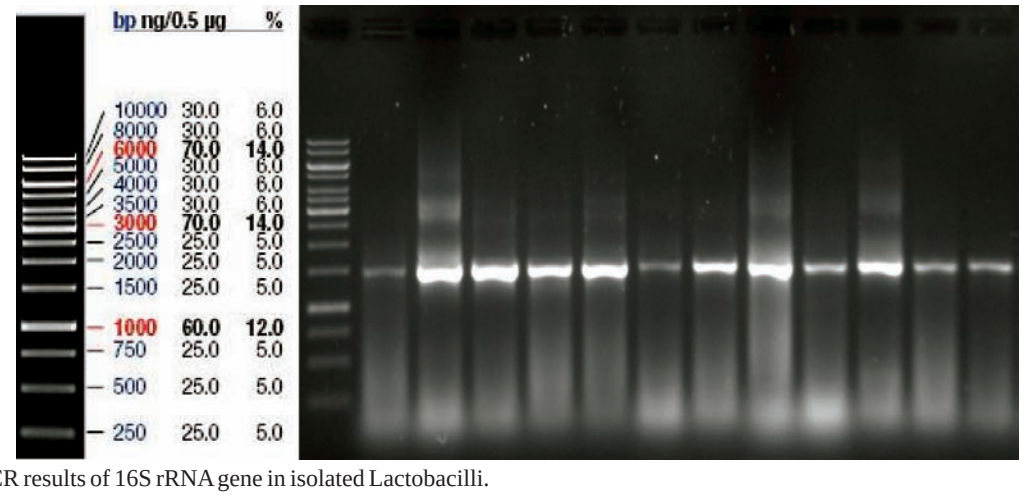

Figure 1. PCR results of 16S rRNA gene in isolated Lactobacilli.

Figure 2. Digestion of the PCR products using TaqI and its comparison with digestion patterns produced by bioinformatic analysis of 16S rRNA sequences obtained from NCBI.

Isolates LB1 and 5 were thoroughly resistant to acid, however LB2, 4, 10 and 11 had intermediate resistance. LB3, 6, 8 and 9 resistance to acidic conditions was weak while only LB12 was destroyed completely.

The results of bile acids tolerance for 12 Lactobacilli isolates have been shown in Table 2.

All isolates were resistant to bile acids except LB8.

The results from Lactobacilli tolerance to different concentrations of salt in 37°C has been shown in figure 4.

Protease and lipase activity: Proteolytic activity was monitored at 3 time points of 24, 48 and 72 h and

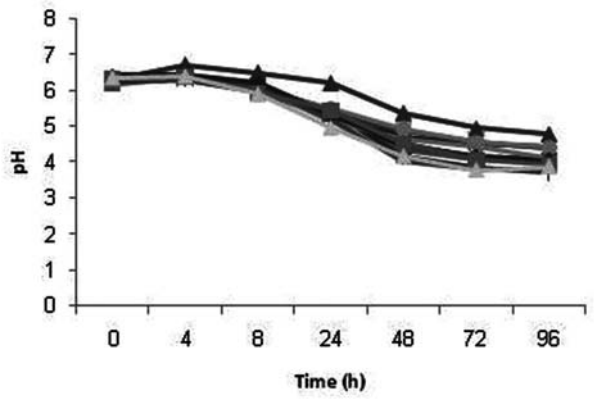

Figure 3. Changes in pH of LB cultures in milk incubated at 37°C.

in 2 temperatures of 30 and 37°C. In 30°C and 37°C, 7 (LB1, 2, 4, 5, 7, 8 and 12) and 9 (LB1, 2, 4, 5, 6, 7, 8, 9, 10 and 12) isolates had proteolytic activity, respectively. This is while PCR results indicated that 10 isolates were positive for protease gene (figure 5).

Lipolytic activity was also monitored at 3 time points of 24, 48 and 72 h and in 2 temperatures of 30 and 37°C. In 37°C, all Lactobacilli exhibited lipolytic properties, while at 30°C, only one (LB5) did not.

Antibiotic resistance: Susceptibility or resistance of the 12 positive Lactobacilli isolates was studied to 8 antibiotics using disk diffusion method, the results of which have been depicted in Table 1.

12, 7, 3, 2 and 1 isolates were sensitive to erythromycin, ampicillin, tetracycline, amikacin and rifampicin, respectively.

Table 1. Number (Cfu mL^{-1}) of colonies before and after acid exposure (pH 1.55) in 37 °C for the 12 Lactobacilli.

Isolates	Time	
	Before	After
LB1	896×10^7	144×10^7
LB2	333×10^6	88×10^5
LB3	987×10^7	1810
LB4	109×10^5	66×10^3
LB5	367×10^7	109×10^7
LB6	375×10^5	340
LB7	981×10^7	141×10^3
LB8	653×10^5	430
LB9	945×10^7	70
LB10	131×10^7	91×10^5
LB11	247×10^7	183×10^3
LB12	270×10^7	0

Discussion

The flavor of cheese has been attributed to the heterofermentative activity of LAB which results in production of acetic acid, fumarate and gas (Hertel, et al., 1991). The specific characteristics of different types of cheese probably get back to the variability of genus and its survival (Gobbetti, Corsetti, Smacchi, Zocchetti, & De Angelis, 1998). Thus, studying the native LAB composition of the traditional cheese may provide us with attractive resources for identification of safe cheese starters with industrial potential. Indeed, Barzegari et al. recently proposed that probiotics be isolated from native microbiomes (Barzegari & Saei, 2012). Native probiotics are supposed to have better therapeutic functions in the native population (Barzegari & Saei, 2012).

Dokhani et al. have isolated L. plantarum and L. casei from Lighvan cheese (Personal communication). In our study, Gram staining and catalase tests along with PCR analysis of 16S rDNA gene demonstrated that 12 traditional Lighvan cheese samples were positive for presence of Lactobacilli. 10 samples belonged to L. plantarum while 2 of them were characterized as L. bulgaricus.

Most of the isolates were shown to grow better in 30 and 37°C than in 40 and 42°C (data not shown).

LABs have been reported as the most common species found in cheese, since they can grow under extreme selective conditions (Centeno, Cepeda, & Rodriguez-Otero, 1996; Pérez Elortondo, Aldámiz Echobarria, Albisu, & Barcina, 1998). Balanced proteolytic properties also contribute to production of interesting organoleptic qualities (Torres-Llanez, Vallejo-Cordoba, Díaz-Cinco, Mazorra-Manzano, & González-Córdova, 2006). Since acid tolerant LAB increase in number at the end of the fermentation process, the favorable proteolytic properties result in favorable organoleptic characteristics of the final product (O'Sullivan, Ryan, Ross, & Hill, 2003). Poveda et al. have reported that the flavor of cheese produced using L. plantarum is better than other industrial cultures (Poveda, Sousa, Cabezas, & McSweeney, 2003). In our experiments, although 10

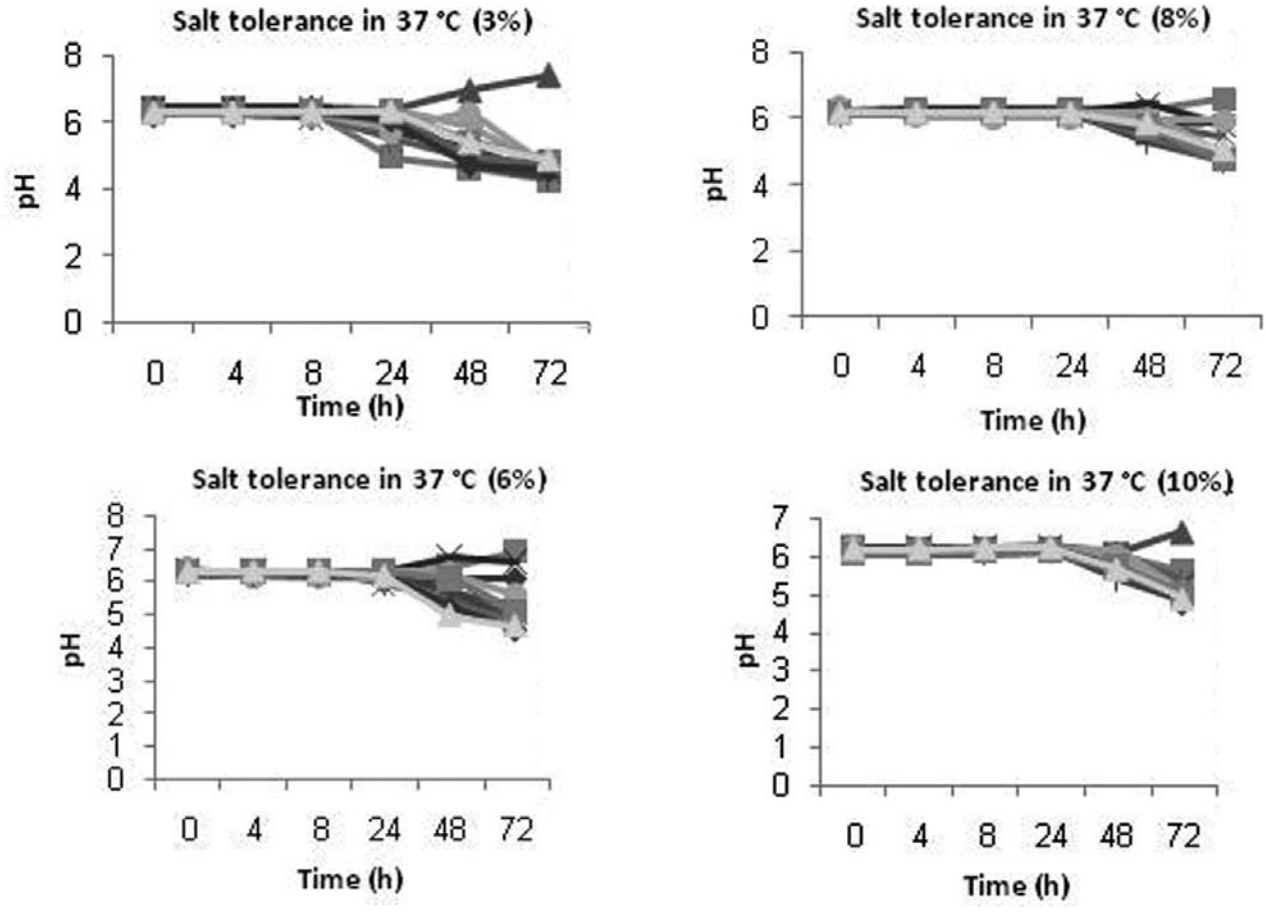

Figure 4. Changes in pH during salt tolerance (3, 6, 8 and 10%) in 37°C for the 12 positive isolates.

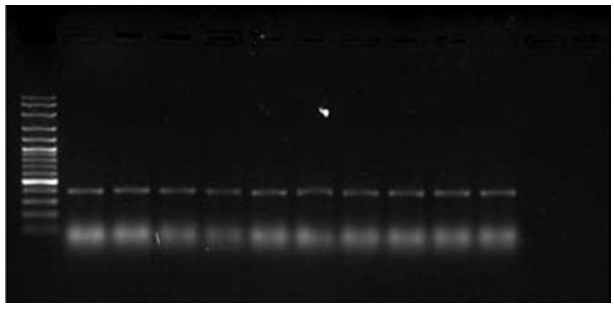

Figure 5. PCR analysis of the protease gene; 10 Lactobacilli are positive for protease while 2 at the right are negative.

Lactobacilli were shown to be protease positive by PCR analysis, in experiments, 9 were protease positive at 37°C and 7 were positive at 30°C. Some isolates have previously been shown to contain protease genes, but no proteolytic activity; the gene may not be expressed in these bacteria (Sánchez, Seseña, Poveda, Cabezas, & Palop, 2005). On the other hand, some LAB do not contain protease gene but possess proteolytic functions (Klijn, et al., 1995). This can explain why some of the LABs in the current study were shown to be protease-positive by PCR, but had no proteolytic activity. The isolates were also screened for lipase activity. Results indicated that all isolates were lipase positive at 37°C, but only one was lipase negative at 30°C.

All isolates were tolerant to 3% NaCl and 9 even tolerated 6-8% NaCl. This is an interesting technological quality, since LAB should be tolerant to the extreme salt concentrations in cheese.

Two isolates were absolutely tolerant to acidity (pH 1.55) and four isolates had intermediate resistance. Four isolates were weakly tolerant and one isolate was very sensitive. All but one isolate were tolerant to 0.03% bile acid. Both acid and bile resistance are among the most important criteria that

Table 2. Optical density 600 of cultures exposed to bile acids in 37°C in different time points for 12 Lactobacilli.

Isolates	Cultures	Time (h)							
		0	1	2	3	4	5	6	24
LB1	MRS	1.66	1.25	1.30	1.32	1.42	1.49	1.68	2<
	MRS+Bile	1.69	1.41	1.48	1.49	1.66	1.68	1.68	1.63
LB2	MRS	0.96	0.94	0.95	0.85	0.90	0.88	0.92	1.64
	MRS+Bile	1.01	0.96	0.95	0.91	0.94	0.89	0.90	0.91
LB3	MRS	0.93	0.90	0.92	0.87	0.94	0.88	0.95	1.21
	MRS+Bile	0.98	0.95	0.98	0.96	0.95	0.89	0.91	0.94
LB4	MRS	0.89	0.90	0.90	0.84	0.84	0.84	0.84	0.86
	MRS+Bile	1.05	0.94	0.98	0.92	0.94	0.85	0.87	1.24
LB5	MRS	1.39	1.19	1.15	1.18	1.32	1.40	1.48	2<
	MRS+Bile	1.51	1.32	1.24	1.26	1.64	1.31	1.28	1.46
LB6	MRS	1.53	1.11	1.19	1.36	1.60	1.69	1.71	2<
	MRS+Bile	1.47	1.24	1.28	1.22	1.29	1.58	1.57	2<
LB7	LB4MRS	1.54	1.32	1.27	1.36	1.29	1.69	1.73	2<
	MRS+Bile	1.81	1.85	1.83	1.72	1.90	2	2	1.94
LB8	MRS	1.14	0.95	1.01	1.98	0.98	1	1.21	2<
	MRS+Bile	1.85	0.92	0.93	0.90	0.93	0.90	0.91	0.95
LB9	MRS	1.57	1.45	1.29	1.35	1.39	1.62	1.72	2<
	MRS+Bile	1.90	1.84	1.81	1.72	1.76	2	1.93	1.80
LB10	MRS	0.88	0.90	1.81	0.85	0.86	0.87	0.95	1.27
	MRS+Bile	0.97	0.97	0.91	1.20	0.97	0.92	0.93	0.99
LB11	MRS	1.72	1.45	1.50	1.57	1.60	1.79	1.84	2<
	MRS+Bile	1.79	1.64	1.62	1.66	1.67	1.64	1.60	1.47
LB12	MRS	1.72	1.38	1.42	1.47	1.56	1.57	1.64	2<
	MRS+Bile	1.95	1.85	1.87	1.74	1.85	1.95	1.93	1.24

Table 3. Results of antibiotic resistance in 12 Lactobacilli.*S=sensitive, R=resistant and I=intermediate resistance.

	Isolates											
Antibiotics	LB1	LB2	LB3	LB4	LB5	LB6	LB7	LB8	LB9	LB10	LB11	LB12
Ampicillin (10 µg)	R	S	S	S	I	I	S	S	S	S	R	R
Amikacin (30 µg)	S	S	R	R	R	R	R	R	R	R	R	R
Erythromycin (15 µg)	S	S	S	S	S	S	S	S	S	S	S	S
Norfloxacine (10 µg)	R	R	R	R	R	R	R	R	R	R	R	R
Penicillin G (10 UI)	R	R	R	R	R	R	R	R	R	R	R	R
Rifampicin (30 µg)	R	R	I	R	I	R	S	R	R	R	I	I
Streptomycin (10 µg)	R	R	R	R	R	R	R	R	R	R	R	R
Tetracycline (30 µg)	R	R	S	I	I	I	S	I	I	S	I	I

should be considered when selecting probiotic bacteria (Barzegari & Saei, 2012). In selection of bacteria to be incorporated into fermented products, antibiotic resistance is among other factors that also

need to be taken into account (Mathur & Singh, 2005). Some of the isolates were shown to be sensitive to erythromycin, ampicillin, tetracycline, amikacin and rifampicin and resistant to norfloxacine, streptomycin and penicillin G.

Results obtained from this study indicate that most of the isolated Lactobacilli can be used as technologically and industrially interesting cheese starters and/or as probiotic candidates to be incorporated into functional foods and products.

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جدا سازی و مطالعه ویژگی‌های پروبیوتیکی و تکنولوژیکی لاکتوباسیلوس‌های موجود در پنیرهای منطقه لیقوان

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چکیده

زمینه مطالعه: پروبیوتیک‌ها بخصوص لاکتوباسیلوس ها از مهمترین میکروارگانیسم‌هائی هستند که در صنعت غذا مورد استفاده قرار می‌گیرند. جداسازی وشناسائی پروبیوتیک‌ها از فرآورده‌های محلی امر ضروری می‌باشد. **هدف:** هدف از تحقیق حاضر جداسازی سویه‌های لاکتوباسیلوس از پنیر های سنتی لیقوان و مطالعه ویژگی های تکنولوژیکی و پروبیوتیکی آنها جهت استفاده بعنوان مایه ی کشت بود. **مواد و روش کار:** برای این منظور ۱۵ نمونه از پنیرهای سنتی لیقوان بطور تصادفی انتخاب گردید. ابتدا سویه‌های لاکتوباسیلوس بر اساس روش کشت و خصوصیات بیوشیمیائی جدا سازی و سپس براساس روش PCR روی ژن 16S rRNA مورد تایید قرار گرفت. خصوصیات پروبیوتیکی جدایه‌ها بر اساس مقاومت در مقابل اسید و صفرا ارزیابی شد. حساسیت جدایه‌ها نسبت به ۸ آنتی بیوتیک مهم مطالعه شد. خصوصیات تکنولوژیکی آنها بر اساس قدرت تولید اسید، میزان تحمل غلظت نمک، همچنین میزان فعالیت پروتئولیتیکی و لیپولیتیکی جدایه‌ها نیز مورد ارزیابی قرار گرفت. **نتایج:** در مجموع ۱۲ سویه لاکتوباسیلوس جدا سازی شد که ۱۰ جدایه مربوط به گونه‌های مختلف لاکتوباسیلوس پلانتاروم و دو جدایه مربوط به لاکتوباسیلوس بولگاریکوس بود. تمامی جدایه ها نسبت به ۳٪ و ۹ جدایه نسبت به ۶-۸٪ نمک مقاوم بودند. دو جدایه بصورت کامل، ۴ جدایه بصورت متوسط، ۴ جدایه بصورت ضعیف نسبت به pH برابر ۱/۵۵ مقاوم بوده و ۱ جدایه حساس بود. به غیر از یک مورد همه جدایه ها نسبت به غلظت ۰/۰۳٪ از املاح صفراوی مقاوم بودند. تعدادی از جدایه‌ها نسبت به اریترومایسین، آمپی سیلین، آمیکاسین وریفامپین حساس بودند. **نتیجه‌گیری نهایی:** نتایج این تحقیق نشان دادکه اکثر اکتوباسیلوس های جداشده از پنیر لیقوان می توانند به عنوان مایه های کشت و یا پروبیوتیک در تولید غذاهای عملکردی مورد استفاده قرار گیرند.

واژه‌های کلیدی: لاکتوباسیلوس، ویژگی‌های پروبیوتیکی، ویژگی‌های تکنولوژیکی، پنیر لیقوان.

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