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Phenotypic and phylogenetic characterization of *Lactobacillus* species isolated from traditional Lighvan cheese

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Abstract

Lighvan cheese (Lighvan panir) is among the most famous traditional cheese in Iran for its desired aroma and flavor. Undoubtedly, the lactic acid bacteria especially the genus *Lactobacillus* are the critical factors in developing the aroma, flavor, and texture in Lighvan cheese. In this study, the Lactobacillus population of the main Lighvan cheese was investigated. The Lactobacillus of the main Lighvan cheese was isolated using specific culture methods according to previously published Guidelines. Then, the phylogenetic features were investigated and the phenotypic characteristics were examined using specific culture methods. Twenty-eight Gram-positive bacterial species were identified belonged to the genus Lactobacillus. According to the same sequences as each other, three groups (A, B, and C) of isolates were categorized with a high degree of similarity to *L. fermentum* (100%) and L. casei group (L. casei, *L. paracasei*, and *L. rhamnosus*) (99.0 to 100%). Random amplified polymorphic DNA (RAPD) fingerprint analysis manifested the presence of three clusters that were dominant in traditional Lighvan cheese. Cluster I was divided into 4 sub-clusters. By the result of carbohydrate fermentation pattern and 16S rRNA sequencing, isolates were identified as *L. rhamnosus*. The isolates in clusters II and III represented *L. paracasei* and *L. fermentum*, respectively as they were identified by 16S rRNA sequencing and fermented carbohydrate patterns. Our result indicated that the specific aroma and flavor of traditional Lighvan cheese can be related to its *Lactobacillus* population including *L. fermentum*, *L. casei, L. paracasei*, and *L. rhamnosus*.

Keywords: Lighvan cheese, *Lactobacillus*, 16S rRNA, RAPD fingerprints

Introduction

Lactic acid bacteria (LAB) have great economic value for the dairy and other fermented food industries. The fermentation of food products is the result of growth association and the interaction among various LAB. Identification of these bacteria is essential in both basic and applied research (Sharma et al. 2020). *Lactobacillus* is one of the most important genera of lactic acid bacteria. The genus *Lactobacillus* is a phenotypically heterogeneous group of Gram-positive, facultatively anaerobic, catalase-negative, non-spore-forming, rod-shaped organisms producing lactic acid as a major end-product of the metabolism (Du et al. 2019). *Lactobacillus* species generally maintain DNA with a low G + C content (approx. 33–53 mol%). Lactobacilli have great commercial significance due to their use in the production of a range of fermented dairy, meat, and vegetable products. There is also a growing interest in their use as probiotics (i.e. dietary adjuncts for man and animals) (Patrignani et al. 2020; Wang et al. 2020). Nucleotide base sequences of *Lactobacillus* 16S ribosomal RNA (rRNA) prepare an accurate basis for phylogenetic analysis and identification (Awd et al. 2020). Random amplified polymorphic DNA (RAPD) is another

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appropriate technique especially for LAB that has been established and successfully used. RAPD is a PCR-based technique, which has been applied for intra- and inter-species genomic distinction of diverse bacteria (Arenas & Salazar 2019; Hu et al. 2020).

Lighvan cheese (Lighvan panir) is the most famous traditional cheese in different regions of Azerbaijan, Iran. This cheese is mainly manufactured from fresh sheep milk with mixtures of approximately 20–30% goat's milk. It's characterized by its white color, pleasant aroma, soft and brittle texture with numerous holes. It has a salty and specific acidic taste making it different from the other cheeses (Alirezalu et al. 2019; Habibi Najafi, 2020).

In the current study, we have focused on the identification of *the Lactobacillus* population to find that if the specific taste of Lighvan cheese is related to exclusive species of *the Lactobacillus* genus. Undoubtedly, the lactic acid bacteria especially the genus *Lactobacillus* are the most important factors in developing the aroma, flavor, and texture in different fermentation products, particularly in Lighvan cheese. These bacteria affect the texture and flavor of the product during growth and reproduction by consuming nutrients in the environment and releasing the final metabolites. In this study, phenotypic characteristics of the *Lactobacillus* isolates and the results of a comparative 16S rRNA phylogenetic analysis are reported. In the next step, the RAPD-PCR method was used as an effective tool for the identification and classification of *Lactobacillus* species isolated from traditional fermented dairy products.

Materials and methods

Materials

Traditional Lighvan cheese was obtained from the native people of Lighvan village (Tabriz, Iran) making it in its traditional form MRS broth and agar medium were procured from Merck Co. (Darmstadt, Germany), and soluble starch was bought from Difco (Detroit, USA). PCR buffer, dNTP, Taq DNA polymerase, and QIAquick kit were procured from Qiagen Co. (Shanghai, China). Gelatin and other analytical grade solvents and reagents were purchased from Sigma-Aldrich (St. Louis, USA). The gradient master cycler (Eppendorf, Germany) was employed for PCR.

Isolation and growth conditions

The lactobacilli characterized in this report were isolated from fermented dairy products including traditional Lighvan cheese. For this purpose, 25 g of each sample was weighed specifically and homogenized with 225 ml of sodium citrate using a stomacher (Seward Stomacher 400 Circulator, UK) at 45 °C for 1 min. The sample was then diluted (1:10) using sterile peptone water and 0.1 ml of each dilution was cultured on Man Rogosa and Sharpe (MRS) agar (Merck, Germany) used for isolating lactic acid bacteria (Milani et al. 2017). Isolates were grown in MRS broth as a common medium for Lactobacilli. The cultures were seeded on MRS agar, and then plates were incubated at 37 °C for 24 h in anaerobic conditions (Coeuret et al. 2003).

Morphological and phenotypical characterization

The first step of determination was carried out based on the cultural and morphological features, physiological characteristics, and specific biochemical tests of the isolates according to the procedures described in Bergey's Manual of Systematic Bacteriology (Bergey et al. 1984). All isolates were tested for Gram reaction, catalase production, and oxidase test. The morphology and features of colonies were also examined on MRS agar medium. Ammonia production from arginine was carried out using the methods of Edwards et al. (1991). Utilization of starch and gelatin were appointed in MRS agar supplemented with 1% soluble starch or 12% gelatin, then the cultures were incubated for 2 days at 37 °C. Hydrolysis of starch was studied by exposing the starch plates to Iodine vapor while the gelatin plates were submerged with 20% trichloroacetic acid. The reduction of nitrate to nitrite was examined in a medium including (per liter): peptone: 5 g, KNO_3: 1.5 g, and NaCl: 6.8 g. Incubation of the cultures was done for 1 week at 35 °C. Urease test was tested on a medium containing (per liter): peptone: 1 g, NaCl: 5 g, KH_2PO_4: 2 g, glucose: 1 g, urea: 20 g, agar: 15 g, and phenol red: 0.1 g. Then the isolates were incubated for 3 days at 37 °C. Gas production from the glucose was detected using Durham's tubes in MRS broth supplemented with 1% glucose. Tubes were incubated for 2 days at 37 °C. The anaerobic condition was prepared by dropping sterile liquid paraffin in each tube after inoculation. The capability of various carbohydrates fermentation was identified on MRS broth medium without meat extract and glucose. The carbon source was added as a filter-sterilized solution to a final concentration of 1% to the sterile basal medium. Carbohydrates utilization was investigated at 24 and 48 h. All of the isolates were examined for fermentation of the various sugars such as arabinose, cellobiose, esculin, D-fructose, galactose, D-glucose, and lactose.

Molecular identification

Genomic DNA extraction was extracted based on the protocol described by Corbin et al. (2001), with slight modifications. The 16S rRNA genes were amplified by polymerase chain reaction (PCR) using universal primers Hal 6F and Hal 6R (Table 1). The reaction mixtures (50 μl) contained 5 μl of 10x PCR buffer, 0.2 mM of dNTP, 1.0 mM $MgCl_2$, 40 pmol of each primer, 4.0 U

Table 1 Oligonucleotide primers used for 16S rRNA sequencing

Primer	Sequence 5′- 3′	Reference
Hal 6F	AGAGTTTGATC(AC)TGGCTCAG	(Karlson et al., 1993)
Hal 6R	TACCTTGTTAGGACTTCACC	(Karlson et al., 1993)
Lac R	AATCCGGATAACGCTTGCC	This study

Taq DNA polymerase, and 50 ng.μl^{-1} genomic DNA as the template DNA. Amplification was carried out in the gradient master cycler as follows: the initial denaturized temperature at 95 °C for 5 min, in the following 35 cycles including denaturized temperature at 95 °C for 60 s, annealing for 50 s, and elongation at 72 °C for 85 s, which was proceeded by a terminal 10 min extension step at 72 °C. DNA fragments were extracted from the gel using a QIAquick kit. The 16S rRNA genes were sequenced by Macrogen Co., Seoul, Korea, and edited with Chromas 2.01. Then, the sequences were compared with representative sequences in the Gene bank database using the BLAST algorithm (https://www.ncbi.nlm.nih.gov/) and their similarities were calculated using the EzTaxon-e server (EzTaxon server 2.1) (Chun et al. 2007). The multiple sequencing was aligned using program CLUSTALX (version 1.83) (Thompson et al. 1997). The phylogenetic trees were designed in the neighbor-joining (NJ) methods using MEGA 4.0 (Saitou & Nei 1987). The nucleotide sequences of the 16S rRNA described in this report were deposited in GenBank with accession numbers: GU168730, GU197389, and GU197388 for the isolates RL10 (group A), RL 40 (group B), and RL 50 (group C), respectively.

The primers applied for the RAPD analysis are listed in Table 2. The reaction mixtures (50 μl) including 5 μl of 10x PCR buffer (Tris-HCl, pH 8.8; 100 mM, KCl; 0.1%, and Triton X-100: 500 mM), deoxynucleoside triphosphate (dNTP): 0.2 mM, $MgCl_2$: 1.0 mM, 50 pmol of each primer, 4 U Taq DNA polymerase, and 25 ng of genomic DNA solution. The amplification was performed in the same cycler as described before, as follows: the initial denaturized temperature at 94 °C for 4 min, in the following 35 cycles including denaturized temperature at 94 °C for 60 s, annealing at 40 °C for 40 s, and elongation at 72 °C for 60 s, and a terminal extension step at 72 °C for 10 min. The PCR amplification products were separated by running on 2% agarose gel. A 1 kb DNA ladder (fermentase) was used as a reference.

Table 2 The primers were applied for the RAPD analysis

Primer	Sequence 5′- 3′	Reference
P8	GTCCCGTTAC	(Abbo et al., 1991)
P10	TTTCACATGG	(Abbo et al., 1991)
OPB-10	CTGCTGGGAC	(Bieliková et al., 2002)
OPF-11	TTGGTACCCC	(Bieliková et al., 2002)
CRA 23	GCGATCCCCA	(Daud Khaled et al., 1997)
OMP-05	GGGAACGTCT	(Gancheva et al., 1999)
OPA-02	TGCCGAGCTG	(Gancheva et al., 1999)
OPL-02	TGGGCGTCAA	(Van Reenen & Dicks, 1996)
OPL-05	ACGCAGGCAC	(Van Reenen & Dicks, 1996)

Results and discussion

Lighvan cheese is a semi-soft cheese that is very popular because of its desirable flavor. However, the main Lighvan cheese is produced in the green village located at the foothills of Mount Sahand southeast of Tabriz, the capital city of East Azerbaijan. This cheese is prepared in a completely traditional way from raw sheep and goat milk, the fermentation also depends on the LAB flora of the raw milk. The natural microbial flora in raw milk, including wild LAB strains, along with natural protease of milk and added rennet, produces a special and desirable flavor of this cheese.

Cheese producers believe that the presence of unique flocks and plants in this region, mountainous climate, and cool air has a favorable effect on lactation and the quality of sheep's milk, and therefore Lighvan cheese has a higher quality than cheese in other regions (Donnelly & Kehler 2016). The production of all fermented dairy products is based on the use of starter cultures such as LAB that initiate rapid acidification of the raw materials and can contribute to microbial safety (Abdi et al. 2006).

Lighvan cheese is stored in tin cans containing saltwater and its ripening period is 3 to 12 months. The best temperature for storing it is 5 or 6 degrees. Moreover, it seems that the salt concentration and specific acidic taste making it different from the other cheese (Alirezalu et al. 2019; Habibi Najafi 2020). The percentage of salt in Lighvan cheese can be between 10 to 15%. Salt is an important substance for cheese production that affects the taste, texture, shelf life, and suitability of cheese (Johnson et al. 2009). Salt is a key supplier of cheese flavor and has been shown to increase the intensity of flavor of ripe cheese (Bae et al., 2017). If more salt is added to the cheese during the production process, the acid production by lactic acid bacteria can be inhibited which causes to increase in the pH and calcium rate of the cheese (Pastorino et al. 2003a, 2003b). Lactic acid bacteria tolerate high concentrations of salt which is the extra benefit compared to the other less tolerant species. This ability helps them to start metabolism for fermentation of lactic acid and production of acid. In addition, their population doesn't allow the proliferation of opportunistic organisms in this condition (Gottschalk 1986). In present study, different species of *Lactobacillus* were isolated from traditional

Lighvan cheese. First morphological, cultural, and carbohydrates fermentation was carried out on them and identified by 16S rRNA gene sequence by phylogenetic analysis. Then RAPD-PCR method was used to classify different species of *Lactobacillus*. All of the mentioned are explained in detail in this section.

Morphological, cultural, and carbohydrates fermentation profile

Forty-five lactic acid bacteria were isolated from traditional Lighvan cheese, after the original characterization of them, 17 were lactic acid cocci and the others were 28 LAB isolates, belonging to the genus *Lactobacillus*. The results showed that all of the isolates were Gram-positive, and catalase/oxidase negative, facultatively anaerobic, non-spore-forming, and rod-shaped organisms. None of the isolates exhibited urease activity or arginine dihydrolase. All isolates failed to reduce nitrate and hydrolyze starch. Five out of the 28 isolates produced acid and gas from glucose, the rest produced acid without gas during fermentation of glucose. The isolates were also tested for the ability to ferment 18 types of carbohydrates. Nineteen isolates demonstrated a very similar fermentation pattern of the carbohydrates and were consequently placed in the group called group A. The other four isolates showed a very close profile of carbohydrate fermentation to each other. The remaining five isolates had very similar fermentation phenotypes. These isolates were located in groups B and C, respectively. Even though members of the B and C groups were not able to ferment esculin, all the isolates in group A, were able to ferment esculin. All of the isolates in group B, fermented cellobiose, while 68% of members of group A, fermented cellobiose. It was noticeable that cellobiose was not fermented by any members of group C. Only 63% of isolates from group A, could ferment arabinose while arabinose was fermented by 25 and 40% of group B and C members, respectively. Five out of 19 isolates in group A produced acid from sorbitol but none of the isolates in group C could ferment sorbitol and only one isolate in group B could not ferment sorbitol. According to fermentation characteristics, the heterofermentative lactobacilli of group A were identified as *L.rhamnosus*. The group B lactobacilli were determined as *L.paracasei*. All isolates in groups A and B, produced acid from mannose and trehalose, while group C members didn't produce it. As melibiose, raffinose and sucrose were catabolized by all of the tested isolates in group C and by none of the 23 isolates in A and B groups, this may help to distinguish group C from others. All of the five mentioned isolates in the last group were identified as *L. fermentum*. They were facultatively heterofermentative lactobacilli which were located in group C. The data are shown in Table 3.

Identification of species by 16S rRNA gene sequence analysis

Comparison of the 16S rRNA gene sequences obtained from *Lactobacillus* isolates was performed with those strains registered in GenBank. Nineteen isolates had the same sequences with each other, as they were already placed in group A by phenotypic tests. The sequence comparison of four isolates demonstrated that all of them had similar sequences, and the other five isolates had the same sequences with each other, as they were already located in groups B and C by biochemical tests. RL 10, RL 40, and RL 50 were the representative isolates of groups A, B, and C, respectively (GenBank accession numbers GU168730, GU197389, and GU197388). The BLAST analysis of the RL 10 nucleotide sequence indicated the highest identity (99% matches) with *L. rhamnosus* (strain: IDCC 3201 with accession number: EF533991). Nucleotide sequence analysis of RL 40 indicated the highest identity (100% matches) with three strains of *L. paracasei* (strains: NRIC 1938, NRIC 1934, and NRIC 0638 with accession numbers: AB362761, AB362760, and AB362702) and three strains of *L. casei* (strains: Shirota, YIT 0209 and YIT 0180 with accession numbers: AB531131, AB008205, and AB008204) and the BLAST analysis of RL 50 nucleotide sequence indicated the highest identity (100% matches) with two strains of *L. fermentum* (strains: NRIC 0147 and NRIC 0135 with accession numbers: AB362628 and AB362616).

Phylogenetic analysis

To construct the phylogenetic position of the three studied isolates, after amplification and determination of the 16S rRNA gene sequence of these isolates, a phylogenetic tree was constructed based on the 16S rRNA gene sequences (about 1500 bp) by the neighbor-joining method. Based on the following phylogenetic analysis, isolates RL 10, RL 40, and RL50, as the representatives of groups A, B, and C, respectively, were placed in the cluster of *the Lactobacillus* genus. The representative isolates of group A (RL 10) and group B (RL 40) were identified as *L. casei* by configuration a very well defined cluster (100% bootstrap) with this species (Fig. 1) but 16S rRNA sequence analysis of RL 10 showed 99% sequence similarity with *L. rhamnosus*, and nucleotide sequence analysis of RL 40 indicated the highest identity with *L. paracasei* and *L. casei* (100% matches). The representative isolate of group C, (RL 50) was placed in the *Lactobacillus* cluster on the phylogenetic tree, with *L. fermentum* as the most closely related species with a bootstrap of 100% (Fig. 1). Also, 16S rRNA sequence analysis of RL 50 indicated the highest identity with *L. fermentum* (100% matches). Furthermore, as reported by Stackebrandt and GOEBEL (1994), 16S rRNA gene sequence analysis is a very good method for identifying

Table 3 Phenotypic characteristics of the isolated *Lactobacillus* species

Group	No. of isolates	Arabinose	Cellobiose	Esculin	D-Fructose	Galactose	D-Glucose	Lactose	Maltose	Mannose	Melibiose	Raffinose	Rhamnose
A	RL5	2	2	2	2	2	2	2	2	2	–	–	–
	RL6	1	2	2	2	2	2	2	1	2	–	–	–
	RL7	2	2	2	2	2	2	2	2	2	–	–	–
	RL9	–	2	1	2	2	2	2	2	2	–	–	–
	RL10	2	–	2	2	2	2	2	2	2	–	–	–
	RL11	–	2	1	2	2	2	2	2	2	–	–	–
	RL13	1	2	1	2	2	2	2	–	2	–	–	–
	RL20	1	–	1	2	2	2	2	1	2	–	–	–
	RL22	–	2	2	2	2	2	2	2	2	–	–	–
	RL23	–	2	1	2	2	2	2	2	2	–	–	–
	RL24	–	2	1	2	2	2	2	2	2	–	–	–
	RL27	1	2	2	2	2	2	2	2	2	–	–	–
	RL42	1	2	2	2	2	2	2	–	2	–	–	–
	RL44	1	2	1	2	2	2	2	2	2	–	–	–
	RL45	–	2	1	2	2	2	2	2	2	–	–	–
	RL46	1	–	1	2	2	2	2	2	2	–	–	–
	RL57	1	–	1	2	2	2	2	1	2	–	–	–
	RL58	–	–	1	2	2	2	2	2	2	–	–	–
	RL64	1	–	2	2	2	2	2	–	2	–	–	–
B	RL21	–	2	–	2	2	2	2	2	2	–	–	–
	RL40	–	2	–	2	1	2	2	–	2	–	–	–
	RL43	–	2	–	2	2	2	2	2	2	–	–	–
	RL56	1	2	–	2	2	2	–	2	2	–	–	–
C	RL26	–	–	–	2	2	2	2	2	–	2	2	–
	RL47	1	–	–	2	–	2	2	2	–	2	2	–
	RL49	–	–	–	2	2	2	2	2	–	2	2	–
	RL50	1	–	–	2	2	2	–	2	–	2	2	–
	RL63	–	–	–	2	2	2	–	2	–	2	2	2

-; negative reaction, 1; weak positive reaction, 2; positive reaction

Table 3 Phenotypic characteristics of the isolated *Lactobacillus* species *(Continued)*

Group	Ribose	Salicin	Sorbitol	Sucrose	Trehalose	Xylose	Catalase	Oxidase	Argenine	Starch	Gelatin	Nitrate	Urea	CO_2
A	2	2	–	–	2	–	–	–	–	–	–	–	–	–
	2	2	–	–	2	–	–	–	–	–	–	–	–	–
	2	2	2	–	2	–	–	–	–	–	–	–	–	–
	2	2	–	–	2	–	–	–	–	–	–	–	–	–
	2	2	–	–	2	–	–	–	–	–	–	–	–	–
	2	2	2	–	2	–	–	–	–	–	–	–	–	–
	2	2	–	–	2	–	–	–	–	–	–	–	–	–
	2	2	–	–	2	–	–	–	–	–	–	–	–	–
	2	2	2	–	2	–	–	–	–	–	–	–	–	–
	2	1	–	–	2	–	–	–	–	–	–	–	–	–
	2	1	–	–	2	–	–	–	–	–	–	–	–	–
	2	2	2	–	2	–	–	–	–	–	–	–	–	–
	2	1	–	–	2	–	–	–	–	–	–	–	–	–
	2	2	–	–	2	–	–	–	–	–	–	–	–	–
	2	2	–	–	2	–	–	–	–	–	–	–	–	–
	2	–	–	–	2	–	–	–	–	–	–	–	–	–
	2	2	2	–	2	–	–	–	–	–	–	–	–	–
	2	2	–	–	2	–	–	–	–	–	–	–	–	–
	2	2	–	–	2	–	–	–	–	–	–	–	–	–
B	2	2	2	–	2	–	–	–	–	–	–	–	–	–
	2	2	1	–	2	–	–	–	–	–	–	–	–	–
	2	2	–	–	2	–	–	–	–	–	–	–	–	–
	2	2	2	–	2	–	–	–	–	–	–	–	–	–
C	2	–	–	2	–	–	–	–	–	–	–	–	–	2
	2	–	–	2	–	–	–	–	–	–	–	–	–	2
	2	2	–	2	–	–	–	–	–	–	–	–	–	2
	2	–	–	2	–	–	–	–	–	–	–	–	–	2
	2	–	–	2	–	–	–	–	–	–	–	–	–	2

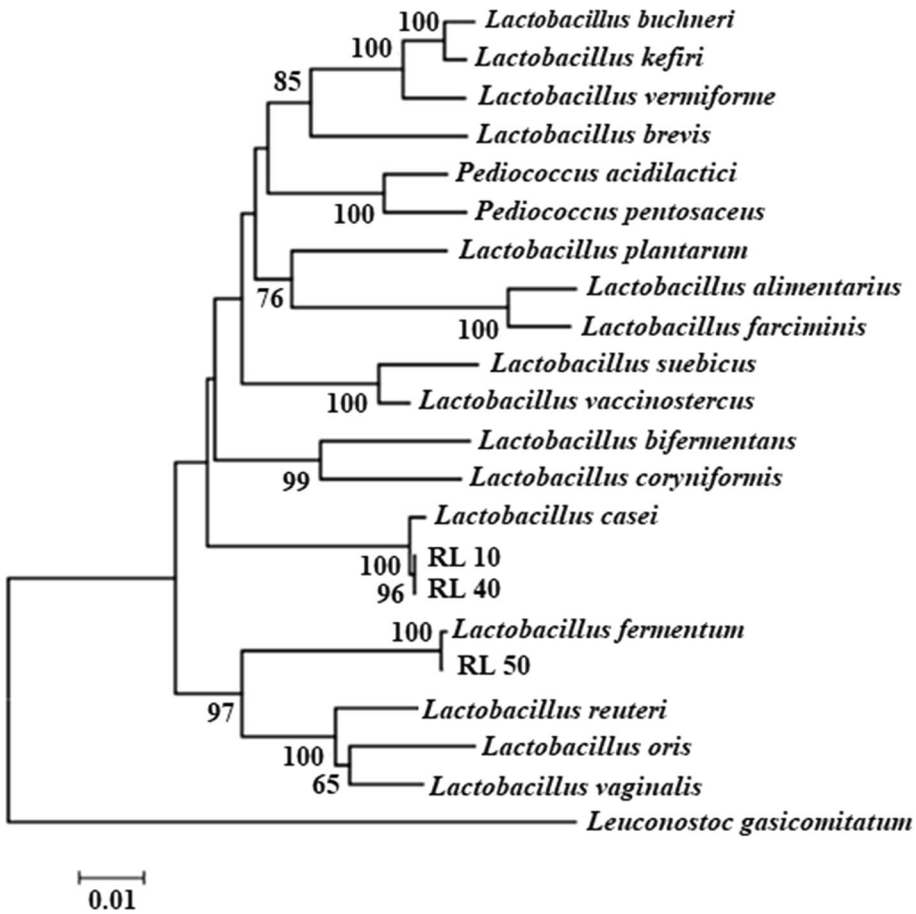

Fig. 1 Phylogenetic tree based on the 16S rRNA gene sequences demonstrated the relationships of RL 10, RL 40 and RL 50 isolates as inferred by the neighbor-joining method. Reference strains used for comparison are shown, *Leuconostoc gasicomitatum* was used as an outgroup. Bootstrap values for a total of 100 replicates are indicated at the nodes of the tree

the organisms at the genus and species level, it cannot distinguish strains at the subspecies level and therefore is not an appropriate method to scale intraspecies relationships. Differentiation of species was less clear due to the high degree of BLAST identity scores between the 16S rRNA sequences of some *L. casei* and *L. paracasei* strains, and also between some *L. rhamnosus* and *L. casei* strains. It is greatly confirmed that *L. casei*, *L. paracasei*, and *L. rhamnosus* belong to the *L. casei* group. They could only be differentiated using phylogenetic analysis of sequences of the 16S–23S large spacer region (Hammes & Vogel 1995) or partial sequences of the *recA* gene (Torriani et al. 2001).

RAPD-PCR profiles

At the first stage nine primers, listed in Table 2, were applied to generate RAPD patterns for the 28 *Lactobacillus* isolates. Among them, five 10-mer oligonucleotide primers were selected to perform RAPD-PCR analysis in the present study. The amplification results performed by primers P8, P10, OPB-10, OMP-05, and OPL-02, were used to calculate the genetic relatedness of the isolates. The dendrogram was constructed by the numerical elaboration of the combined RAPD patterns of five primers based on the presence or absence of the DNA band at a particular location. Genetic relationships between the studied *Lactobacillus* isolates based on the NTSYS-PC software package were determined and the dendrogram was constructed using the UPGMA program. The resulting dendrogram is shown in Fig. 2. The dendrogram described the different species of the *Lactobacillus*. Based on these data, the investigated isolates were diverted into three clusters, mentioned as I, II, and III in Fig. 2. In general, in cluster I the studying isolates were divided into 4 sub-clusters, they are represented as a, b, c, and d in Fig. 2. The isolates located in the sub-cluster a, demonstrated very similar polymorphic DNA bands as well as the isolates placed in sub-cluster c, while sub-clusters b and d showed indistinguishable band patterns from each other and the two above-mentioned sub-clusters. By the result of carbohydrate fermentation pattern and 16S rRNA sequencing, these

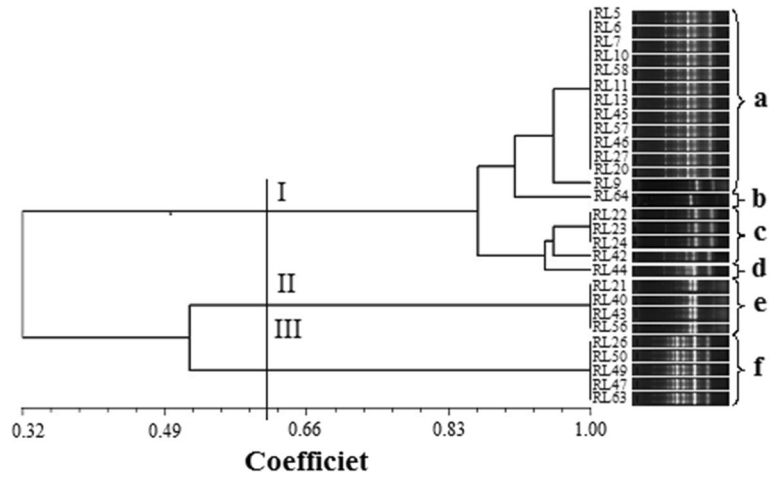

Fig. 2 Cluster analysis of isolated *Lactobacillus* species based on RAPD-PCR. The dendrogram was constructed by using the UPGMA program. RAPD-PCR profiles of the 28 studied LAB isolates were generated with primer P8

isolates were identified as *L. rhamnosus*, though RAPD-PCR analysis could group them into 4 sub-clusters. Cluster II consisted of the group e isolates that showed the same DNA bands and the band patterns of cluster III which contained group f isolates, were the same as well. The studied isolates in clusters II and III represented *L. paracasei* and *L. fermentum,* respectively as they were identified by 16S rRNA sequencing and fermented carbohydrate patterns.

Conclusion

The production of aromatic substances in different cheeses is a result of the metabolic activities of various microorganisms in the cheese. Undoubtedly, lactic acid bacteria are one of the most important factors in the formation of aroma, taste, and texture in various fermented products, including liqueur cheese, the most important feature is the production of lactic acid. On the other hand, the dairy industry is looking for new approaches to increase the variety of products. By examining the native lactic acid microorganisms present in raw milk products, it may be possible to produce new products with a broader range of aromatic compounds and high affinity. This study aimed to identify different species of *Lactobacillus* isolated from Lighvan cheese. Bacteria were evaluated after isolation with techniques based on phenotypic and genotypic methods.

The morphological, cultural, carbohydrates fermentation profile, 16S rRNA gene sequencing, and RAPD-PCR analysis confirmed that the studied isolates belong to *L. rhamnosus, L. paracasei, and L. fermentum*. Despite the similar 16S rRNA sequences, RAPD-PCR analysis divided *L. rhamnosus* into 4 subgroups. Although the 16S rRNA gene sequence had the efficiency to distinguish the *Lactobacillus* species, it may not be a comprehensive method to differentiate the members of the *L. casei* group (*L. casei*, *L. paracasei*, and *L. rhamnosus*). Our result indicated that the specific aroma and flavor of traditional Lighvan cheese can be related to its *Lactobacillus* population including *L. fermentum*, *L. casei, L. paracasei*, and *L. rhamnosus.* These results can be interpreted as meaning that the specific flavor of the traditional Lighvan cheese may be is due to the population of *Lactobacillus* in the natural flora as mentioned above. Also, studies demonstrated that the use of 16S–23S rRNA intergenic spacer region (ISR) and partial sequences of the *recA* gene can be a valuable step for more accurate identification of the lactobacilli population.

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Authors' contributions

HF and SZV performed the experiments; HF and VT analyzed of data; HF, HA, MSH, and VT prepared the manuscript; HF and HA wrote and edited the manuscript; MSH designed the experiments; MSH led and supervised the project. The author(s) read and approved the final manuscript.

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Availability of data and materials

The authors confirm that the data supporting and the findings of this study are available within the article.

Declarations

Ethics approval and consent to participate

There is no involvement of humans or animals in this study.

Consent for publication

All other authors declare no conflict of interest.

Competing interests
We declare that we have no significant competing financial, professional, or personal interests that might have influenced the performance or presentation of the work described in this manuscript.

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