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Full Length Research Paper

Identification of *Lactobacillus pentosus*, *Lactobacillus paraplantarum* and *Lactobacillus plantarum* in Lighvan cheese with 4 month ripening period by means of *rec*A gene sequence analysis

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***Lactobacillus plantarum* species, one of the principal group of the facultatively heterofermentative lactobacilli (FHL) in the non-starter microbial flora isolated from most cheeses was characterized in the Lighvan cheese with 4 month ripening period. In this study, we succeeded in differentiating *Lactobacillus pentosus*, *Lactobacillus paraplantarum* and *Lactobacillus plantarum* by *rec*A gene sequencing comparison. The sizes of amplicon were 318 bp for *L. plantarum*, 218 bp for *L. pentosus* and 107 bp for *L. paraplantarum*. Based on results obtained in this investigation using *rec*A gene, 86% of lactobacilli isolates were classified as *L. pentosus* and 14% were classified as *L. plantarum*. Moreover, the development of the FHL in Lighvan cheese varied according to ripening time.**

Key words: Lighvan cheese, *Lactobacillus plantarum*, polymerase chain reaction (PCR), *rec*A gene.

INTRODUCTION

Mesophilic lactobacilli constitute the majority of the non starter lactic acid bacteria (NSLAB) present in most types of cheeses. These lactobacilli commonly include *Lactobacillus plantarum*, *Lactobacillus sake*, *Lactobacillus curvatus*, *Lactobacillus casei*, *Lactobacillus paracasei*, *Lactobacillus pentosus* and *Lactobacillus paraplantarum* (Beresford et al., 2001; Stiles and Holzapfel, 1997), which may have entered into the cheese adventitiously from milk and the immediate surroundings during cheese processing (Mannu et al., 2000).

It is well known that the mesophilic lactobacilli play an important role during the ripening of cheese. The cheese ripening is a very complex and slow biochemical process that involves three primary reactions: Glycolysis, lipolysis and proteolysis (Smit et al., 2005). However, the precise role of these bacteria in cheese flavour development is still equivocal, but it is believed that they may be involved in proteolysis and in amino acid catabolism. Therefore, in recent years, much attention has been focused on them as a means of accelerating cheese maturation, considering the role that their proteolytic system and other hydrolytic enzymes might have in the development of cheese flavor and texture (Smit et al., 2005).

NSLAB including the earlier mentioned bacteria, reach a high number of viable cells during ripening. In Comté and Cheddar cheeses, the initial small population of occasional NSLAB ultimately becomes the dominant bacterial population in matured cheese (Berthier et al., 2001; Peterson and Marshall, 1990). It appears therefore that there is a great need to characterize and preserve mesophilic lactobacilli occurring in large numbers as unintentional microbial flora, particularly in unpasteurized milk cheeses. Lighvan is a kind of traditional semi-hard cheese manufactured in Lighvan village, Tabriz province, Iran, at farmhouse level, from ewe's raw milk using lamb rennet paste and without any addition of selected or

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Abbreviations: FHL, Facultatively heterofermentative lactobacilli; **NSLAB,** non starter lactic acid bacteria; **FTIR,** fourier transform infrared; **RAPD-PCR,** randomly amplified polymorphic DNA-polymerase chain reaction; **AFLP,** amplified fragment length polymorphism.

natural starter culture (Abdi et al., 2006).

Previous studies based on conventional methods performed in this laboratory showed that lactobacilli are the main microbial group colonizing in this cheese (Abdi et al., 2006). The aim of this study is to identify the species of the lactobacilli based on morphological, biochemical and molecular properties in Lighvan cheese with 4 month ripening.

MATERIALS AND METHODS

Sampling

Eight batches of Lighvan cheese with 4 month ripening period were purchased from the local shops. Ten grams of each sample was homogenized in 90 ml sterile normal saline solution and serial dilutions were made in the same solution and then plated on the sorbitol agar as duplicate plates and incubated at 37°C for 3 days.

Isolation

Culturing of 30 colonies that different in shape, size and diameter on the sorbitol agar plates were chosen at random after doing the gram, spore and catalase tests and microscopic observation. Gram positive, nonsporforming and catalase negative rods were subcultured on MRS at 37°C. The pure cultures were frozen and stored at -80°C in MRS broth containing 50% glycerol for further analysis.

Phenotypic characterization

Gram-positive and catalase negative rods were analyzed for their ability to grow in MRS broth at 15°C for 7 days and at 45°C for 3 days. Also, ability to produce Co_2 from glucose was assayed by subculturing the isolates in MRS broth tubes containing Durham bells. Fermentation of carbohydrates was determined on MRS broth without glucose and meat extract containing bromocresol purple (0.05 gl^{-1}) as a pH indicator and supplemented with 1% of the following carbohydrates: Ribose, lactose, xylose, arabinose, sorbitol, melezitose, melibiose, raffinose, gluconate, mannose, manitol, cellebiose and trehalose at 37°C for 7 days (Peter and Sneath, 1996). Two replicate tests were carried out for each isolated strain.

Genotypic characterization

DNA preparation

Genomic DNA of *Lactobacillus* species was extracted as described by Chagnaud et al. (2001), but with the following modification: lactobacilli cells was resuspended in 500 µl of lysis buffer (4 mg/ml lysozyme; 12% PEG 6000; 10 Mm Tris-Hcl, pH 8).

Species-specific polymerase chain reaction (PCR)

Identification of *L. plantarum* group species was performed by amplification of *rec*A gene. The primers used were paraF (5'-GTC ACA GGC ATT ACG AAA AC-3'), pentF (5'-CAG TGG CGC GGT TGA TAT C-3'), planF (5'-CCG TTT ATG sCGG AAC ACC TA-3') and pREV (5'-TCG GGA TTA CCA AAC ATC AC-3') (Torriani et al., 2001). For PCR amplification, 50 ng of genomic DNA was added to 15 µl PCR mixture containing 1 U $µl^{-1}$ of *Taq* polymerase, 2.5 Mm $Mgcl_2$, 0.2 mM of each dNTP and 1X buffer. PCR was performed with initial temperature at 94°C (3 min), 30 cycles of denaturation at 94°C (30 s), annealing at 56.8°C (10 s) and elongation at 72°C, (10 s). The PCR reaction was terminated at 72°C for 10 min, thereafter cooled to 4°C.

Gel electerophoresis

Gel electerophoresis was carried out by applying 5 µl of the sample to 2% agarose gel. The gels were run for approximately 40 min at 80 V in 1×TBE buffer. Gel was then stained in ethidium bromide and thereafter washed for 10 min and visualized with an UV transilluminator.

RESULTS AND DISCUSSION

Results of the total count of lactobacilli on the sorbitol agar showed that a remarkable number of lactobacilli existed in the cheese samples. These results were compared to results of Abdi et al. (2006) which showed that the total count of lactobacilli was the highest among the other isolates. The aim of this project was not to identify the proportion of lactobacilli count to the total NSLAB count. Therefore, the culture was made on the lactobacilli selective agar just to isolate the genus of *Lactobacillus.* Results of the preliminary characterization of *L. plantarum* species isolated from Lighvan cheese with 4 month ripening period performed using phenotypical tests based on shape, diameter and color differentiation and after microscopic assay, are shown in Table 1.

According to the Bergey's manual, all the rod shape, gram positive, catalase negative and nonsporforming isolates were related to the genus *Lactobacillus* (Table 1) (Kandier and Weiss, 1996). In addition, since all isolates could only grow at 15°C, they belonged to the mesophilic lactobacilli group. Also, the pH value of tested fermentation medium was reduced but no gas bubble was seen in the Durham tube. It could therefore be concluded that the isolate belonged to the FHL group. The results of the sugar fermentation performed to identify related species are shown in Table 2.

According to the Bergey's manual, the D1, H5, A3, A5, H4 and C4 isolates were classified as *L. plantarum* group species (Kandier and Weiss, 1996). The genetic heterogeneity of the *L. plantarum* group has been demonstrated by Dellaglio et al. (1975) on the basis of DNA-DNA hybridization data. In their study, three groups were identified and were later classified as *L. plantarum*, *L. pentosus* and *L. paraplantarum*.

Despite the importance of these species for production of plant, animal and fish fermented foods, their precise identification is complicated by ambiguous response of traditional physiological tests which lead to limitation in their industrial applications. The species; *L. plantarum*, *L. pentosus* and *L. paraplantarum* are genotypically closely related and show highly similar phenotypes. For instance, *L. pentosus* can be distinguished from *L. plantarum* by its ability to produce acid from D-xylose and L-arabinose

Table 1. Morphological, cultural and physiological characteristics of the isolated strains.

Characteristic	Property	Strains					
		A3	H5	A5	D1	H4	C4
Colony properties	Color	White	White	White	White	Milky	White
	Shape	Amorphous	Lentiform	Lentiform	amorphous	Lentiform	Circular
	Diameter (mm)	3	>1	2.5	2	2	1.5
Cell morphology		Single, pair rounded rods	Single, pair rounded rods	Single, pair rounded rods	Single, pair rounded rods	Single, pair rounded rods	Single, pair rounded rods
Gram strain		+	+	+	+	+	+
Spore formation		-	-	-	-	-	-
Catalase activity		-	-	-	-	-	-
Gas production from glucose		-	-	-	-	-	-
Growth at different temperature	15°C	+	+	+	+	+	+
	45°C	-	-	-	-	-	-

The alphabets represents each cheese batch used in the experiments; +: positive reaction; - : negative reaction.

Table 2. Results of the biochemical characterization of the strains.

Strain	Arabinose	Terhalose	Cellebiose	Gluconate	Ribose	Melezitose	Melibiose	Raffinose	Xylose	Manitol	Mannose	Sorbitol	Lactose
A3	-	+	+	+	+	-	+	+	-	+	+	+	+
H5	-	+	+	+	+	+	+	+	-	+	+	+	+
A5	-	+	+	+	+	+	+	+	-	+	+	+	+
D1	-	+	+	+	+	-	+	+	-	+	+	+	+
H4	-	+	+	+	+	-	+	+	-	+	+	+	+
C4	-	+	+	+	+	-	+	+	-	+	+	+	+

The alphabets represent each cheese batch used in the experiments. +: positive reaction. -: negative reaction.

(Fred et al., 1921). However, these phenotypic characteristics are not sufficient to distinguish *L. pentousu*s from *L. plantarum* since some strains ferment arabinose but not xylose or both. This problem in definite identification of these species has been mentioned by some researchers like Bringel et al. (1996) and Osawa et al. (2000).

In this study, we succeeded in differentiating *L. pentosus*, *L. paraplantarum* and *L. plantarum* by means of *rec*A gene sequencing comparison (Torriani et al., 2001) (Figure 1). The sizes of amplicon were 318 bp for *L. plantarum*, 218 bp for *L. pentosus* and 107 bp for *L. paraplantarum* (Spano et al., 2002). *Rec*A is a small protein (352 amino acids in *Escherichia coli*) implicated in homologous DNA recombination, SOS induction and DNA damage-induced mutagenesis. This panoply of functions implies multiple biochemical activities, including DNA binding (double and single stranded), pairing and exchange of homologous DNA, ATP hydrolysis, and coproteolitic cleavage of the Lex A, Ic I and Umu D proteins (Eisen et al., 1995). Due to its fundamental role, the *rec*A gene is ubiquitous, and its gene product has been proposed as a phylogenetic marker for distantly related species. Some other methods are used by some investigators to distinguish the

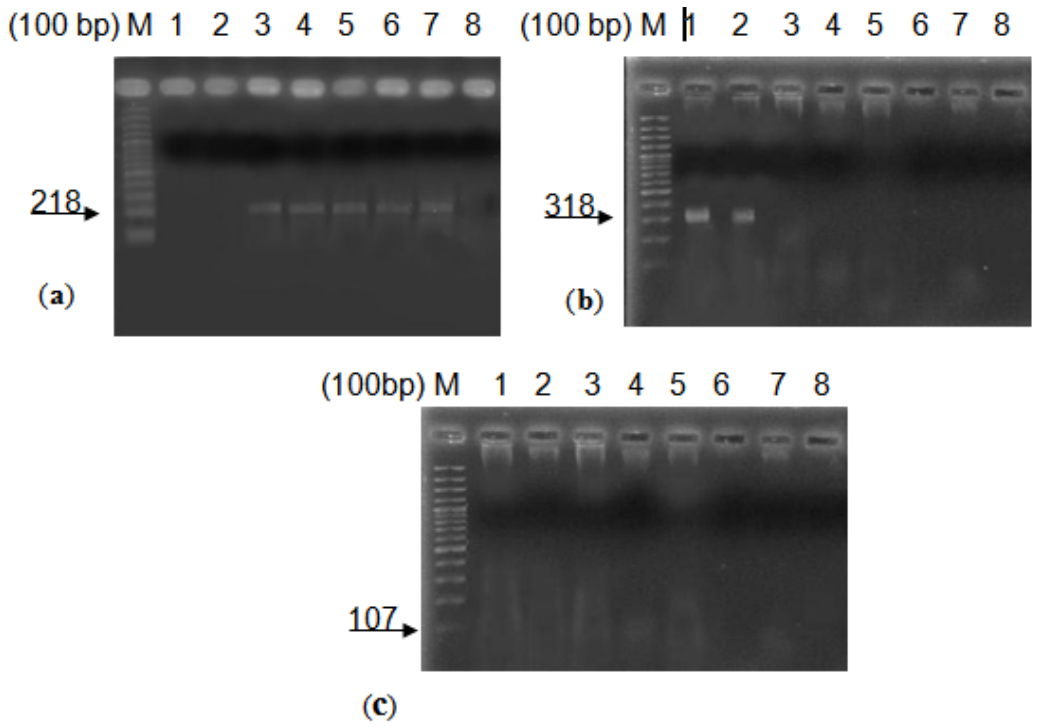

Figure 1. Amplification products obtained from the *rec*A PCR species-specific test. (a) pentF/ pREV primer; (b) planF/ pREV primer and (c) paraF/ pREV primers. M, DNA molecular weight markers (100 bp); lane 1, *L. plantarum* (positive control), lanes 2 to 7: A3, H5, A5, D1, H4 and C4 strains; lane 8, water (negative control).

earlier mentioned species and their comparisons with the application of *rec*A gene.

Curk et al. (1994) showed that due to high similarity in structure, fourier transform infrared (FTIR) spectroscopy of lactobacilli from breweries was not able to differentiate spectra from *L. plantarum* and *L. pentosus*. However, Bringel et al. (1996) and Torriani et al. (2001) could get satisfying results by southern type hybridization bya *pyr DEF* probe, randomly amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) and amplified fragment length polymorphism (AFLP), but such methods are not suitable for routine identification requirements. The difficulty of correct identification of these species and the increasing interest in some of their properties, e.g. probiotic activity (Devries et al., 2006) and tannin degradation (Osawa et al., 2000), indicates the need for a simple, rapid and reliable molecular method for definite differentiation of *L. plantarum, L. pentosus* and *L. paraplantarum* from each other. PCR using species-specific oligonucleotides designed based on phylogenetic molecular markers could be a useful approach, since these molecules are ubiquitous and relatively highly conserved. For this purpose, 16S ribosomal DNA sequences are not suitable because of the high identity value (>99%) shared by *L. plantarum* and *L. pentosus* (Berthier and Ehrlich, 1998).

Consequently, definiteness phylogenetic distances was also not feasible by such a classical approach for *L. plantarum* group species. Therefore, it was proposed that the *rec*A gene could be used as a phylogenetic marker, as it has already given satisfying results for many bacterial genera, including bifidobacteria (Kullen et al., 1997).

On the basis of results obtained in this investigation using *rec*A gene, 86% of Lactobacilli isolates were classified as *L. pentosus* and 14% as *L. plantarum*, but the majority of other studies performed on the other cheese types made from ewe's raw milk like Lighvan cheese showed that the other strains are dominant. For instance, Ostile et al. (2004) investigated microbial flora in the Norwegian semi-hard cheese made from ewe's unpasteurized milk during ripening using biochemical and physiological assays, species-specific PCR and 16S rDNA sequencing, and their results showed that after 3 months of ripening, the NSLAB species *L. paracasei* dominated the other species.

By comparing the results of this work with the earlier results mentioned, it could be concluded that these properties in Lighvan cheese are due to the cheese properties (semi hard with 59% moisture, 4.08% Nacl and pH = 3.9) which are age of cheese, animal feed, ewe's breed and cheese making environmental contamination. Since Lighvan cheese has a special flavor among the Iranians' traditional cheese types, we suggest the conti-

nuation of the investigation of the influence of NSLAB on flavor during the whole ripening period using *rec*A gene.

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