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Identification of *L. pentosus, L. paraplan*tarum and *L. plantarum* in Lighvan cheese with 4 month ripening period by means of *rec*A gene sequence analysis

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***Abstract*—Facultative Heterofermentative Lactobacilli (FHL) are responsible for enhancing unique flavour properties in traditional dairy products such as Lighvan cheese through biochemical mechanisms. In addition to saving domestic genetic resources, industrial production of semi-traditional dairy products is one of the major objectives of non-starter microflora identification in indigenous dairy products. *Lactobacillus plantarum* species, one of the principal groups of the facultatively heterofermentative lactobacilli 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 *L. pentosus, L. paraplantarum* and *L. 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 the results obtained in this investigation using *rec*A gene, 86% of lactobacilli isolates were classified as *L. pentosus* and 14% as *L. plantarum*. Moreover the development of the FHL in Lighvan cheese varied according to ripening time.**

Keywords: Lighvan cheese; Lactobacillus plantarum; PCR; recA gene.

I. Introduction

Mesophilic lactobacilli constitute the majority of the non-starter lactic acid bacteria (NSLAB) present in most types of cheese. These lactobacilli commonly include *Lactobacillus plantarum*, *L. sake*, *L. curvatus*, *L. casei*, *L. paracasei*, *L.pentosus* and *L. paraplantarum* [5; 17]. They may have been entered adventitiously from milk and the immediate surroundings during cheese processing [12]. 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. These bacteria 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 a role in the development of cheese flavor and texture [10]. NSLAB including afore-mentioned bacteria reach a high number of viable cells during ripening. In Comté and Cheddar cheese the initially small population of occasional NSLAB ultimately becomes dominate bacterial population in matured cheese [7, 22]. It appears therefore that there is a great need to characterize and to preserve mesophilic lactobacilli occurring in large numbers as unintentional microbial flora particularly in unpasteurized milk cheese. 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 adding any of the selected or natural starter culture. Previous studies based on conventional methods performed in this laboratory showed that lactobacilli are the main microbial groups colonizing in this cheese [18]. The aim of this study was to identify the species of the lactobacilli based on morphological, biochemical and molecular properties in Lighvan cheese with 4 month ripening.

II. Materials and Methods

A. 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 9o ml sterile normal saline solution and serial dilutions were made in the same solution then plated on the sorbitol agar as duplicate plates and incubated at 37°C for 3 days.

B. Isolation

Cloning of 30 numbers, different in shape, size and diameter on the sorbitol agar plates were chosen at random that 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.

C. 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 [Two replicate tests were carried out for each isolated strain].

D. Genotypic characterization

1) DNA preparation

Genomic DNA of Lactobacillus species was extracted as described by Chagnaud et al. [16], 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).

2) Species-specific 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') [21]. 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, 3min, 30 cycles of denaturation at 94°C (30s), annealing at 56.8°C (10s) and elongation at 72°C, 10s. The PCR reaction was terminated at 72°C for 10 min thereafter cooled to 4°C.

3) Gel electerophoresis

Gel electerophoresis was carried out by applying 5 μ l 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.

III. Results and Discussion

The results of the total count of lactobacilli on the sorbitol agar shows that a remarkable number of lactobacilli existing in the cheese samples. These results were compared to the results of abdi et al. [18] in which it was shown that the total lactobacilli were highest among the other isolates. To the extent that the aim of this project was not to identify the proportion of lactobacilli count to the total NSLAB count, the culture was made on the lactobacilli selective agar just to isolate the genus of lactobacillus. The results of the preliminary characterization of *L. plantarum* species isolated from Lighvan cheese with 4 month ripening period are shown in the Table 1.

TABLE 1. MORPHOLOGICAL, CULTURAL AND PHYSIOLOGICAL CHARACTERISTICS OF THE ISOLATED STRAINS.

Characteristics	Strains					
	A3	***H5***	***A5***	***D1***	***H4***	***C4***
Colony properties (color, shape, diameter)	White amorphous 3 mm	White lentiform, >1 mm	White lentiform, 2.5mm	White amorphous 2 mm	Milky Lentiform 2mm	White circular 1.5 mm
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 in the Tables belongs to each cheese batch used in the experiments.
+: positive reaction
- : negative reaction

According to the Bergey's manual, all the rod shape, Gram positive, catalase negative and nonsporforming isolates were related to the genus *Lactobacillus* (Table1) [15]. In addition, since all isolates could grow only 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 be therefore concluded that the isolates belong to the FHL group. The results of the sugar fermentation performed to identify related species are shown in Table 2.

TABLE 2. RESULTS OF THE BIOCHEMICAL CHARACTERIZATION OF THE STRAINS.

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

According to the Bergey's manual the D1, H5, A3, A5, H4 and C4 isolates are classified as *L. plantarum* group species [15].

The genetic heterogeneity of the *L. plantarum* group has been demonstrated by Dellaglio et al. [9] on the basis of DNA-DNA hybridization data. In their study, three groups identified which were later classified as *L. plantarum*, *L. pentosus* and *L. paraplantarum*.

Despite the importance of these species for the 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 [2]. However, these phenotypic characteristics are not sufficient to distinguish *L. pentousus* from *L. plantarum* because 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. [8] and Osawa et al. [19] In this study, we succeeded in differentiating *L. pentosus*, *L. paraplantarum* and *L. plantarum* by means of *rec*A gene sequencing comparison [21] (Fig. 1). The sizes of amplicon are 318 bp for *L. plantarum*, 218 bp for *L. pentosus* and 107 bp for *L. paraplantarum* [11].

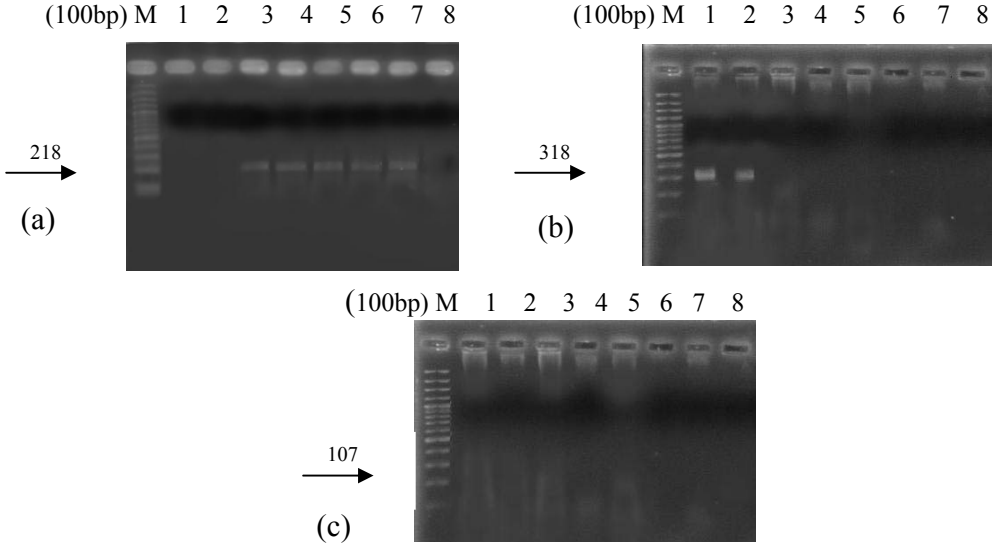

Figure 1. Amplification products obtained from the recA PCR species-specific test. (a) pentF/ pREV primer; (b) planF/ pREV primer and (c) .paraF/ pREV primers. M; DNA molecular weight markers (100 bp), lane 1; *L. plantarum* (positive control), lanes 2-7; A3, H5, A5, D1, H4, and C4 strains. lane 8; water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

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, lc I, and Umu D proteins [1]. Due to its fundamental role, the recA gene is ubiquitous, and its gene product has been proposed as a phylogenetic marker for distantly related species. Some other methods used by some investigators for distinguishing of afore-mentioned species and their comparisons to the application of *rec*A gene are as follows;

Curk et al. [3] 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. [8] and Torriani et al. [21] could get satisfying results by southern type hybridization bya pyr DEF probe, randomly amplified polymorphic DNA-PCR, and 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 [4] and tannin degradation [19], we need a simple, rapid and reliable molecular method for a definite differentiation of *L. plantarum*, *L. pentosus* and *L. paraplantarum* from one another. 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* [6].

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 [13]. Based on the results obtained in this investigation using recA gene, 86% of Lactobacilli isolates are 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 the other strains are dominate. For instance the results obtained from the study carried out by Østile et al. [14] shows that *L. paracasei* dominated.

By comparing the results of this study with the afore-mentioned results, it could be concluded that unique flavor properties in Lighvan cheese are due to the cheese physicochemical properties (semi hard with 59% moisture, 4.08%Nacl and pH=3.9), 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 that other researchers investigate the influence of NSLAB on flavor during the whole ripening period using *rec*A gene.

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REFERENCES

[1] A.J. Eisen, "The RecA Protein as a Model Molecule for Molecular Systematic Studies of Bacteria: Comparison of Trees of RecAs and 16S rRNAs from the Same Species", J. Mol. Evol. vol. 12, Dec. 1995, pp.1105-1123, doi:10.1016/S0169-5347(00)89195-8.

[2] B.E. Fred, H.W. Peterson, and A.J. Anderson, "The characterization of certain pentose-DE-storing bacteria, especially as concerns their action arabinose and xylose", J. Biol. Chem, vol. 40, Aug. 1921, pp. 385-403.

[3] C.M. Curk, F. Peledan, and C.J. Hubert, "Fourier Transform infrared (FTIR) spectroscopy for identifying Lactobacillus specises", FEMS. Microbiol. Lett, vol. 123, Nov. 1994, pp. 241-248, doi: 10.1111/j.1574-6968.1994.tb07231.x.

[4] C.M. Devries, E.E. Vaughan, M. Kleerebezem, and M.W. Devos, "Lactobacillus plantarum-survival, functional and potential probiotic properties in the human intestinal tract", Int. Dairy. J, vol.16, Sep. 2006, pp.1018-1028, doi:10.1016/j.idairyj.2005.09.003.

[5] E.M. Stiles, and H.W. Holzapfel, "Lactic acid bacteria of foods and their current taxonomy", Int. J. Food. Microbiol, vol. 36, Apr. 1997, pp. 1-29, doi:10.1016/S0168-1605(96)01233-0.

[6] F. Berthier, and D.S. Ehrlich, "Rapid species identification within two groups of closely related lactobacilli using PCR primers that target the 16S/23S rRNAspacer region", FEMS. Microbiol. Lett, vol. 161, Apr. 1998, pp. 97-106. doi: 10.1111/j.1574-6968.1998.tb12934.x.

[7] F. Berthier, E. Beuvier, A. Dasen, and R. Grappin, "Origin and diversity of mesophilic lactobacilli in Comté cheese, as revealed by PCR with repetitive and species-speci.c primers", Int. Dairy. J, vol. 11, Jul. 2001, pp. 293-305. doi:10.1016/S0958-6946(01)00059-0.

[8] F. Bringel, C.M. Curk, and C.J. Hubert, "Characterization of Lactobacilli by Southern-Type Hybridization with a Lactobacillus plantarum pyr DFE Probe", Int. J. Syst. Bacteriol, vol. 46, Apr. 1996, pp. 588-594.

[9] F. Dellaglio, V. Bottazzi, and M. "Vescovo Deoxyribonucleic Acid Homology Among Lactobacillus Species of the Subgenus Streptobacterium Orla-Jensen", Int. J. Syst. Bacteriol, vol. 25, Apr. 1975, pp. 160-172.

[10] G. Smit, A.B. Smit, and M.J.W. Engels, "Flavour formation by lactic acid bacteria and biochemical flavour profiling of cheese products", FEMS. Microbiol. Rev, vol. 29, Aug. 2005, pp. 591-610, doi:10.1016/j.femsre.2005.04.002.

[11] G. Spano, L. Beneduce, D. Tarantino, G. Zapparoli, and S. Massa, "Characterization of Lactobacillus plantarum from wine must by PCR species-specific and RADP-PCR", Lett. Appl. Microbiol, vol. 35, Nov. 2002, pp. 370-374, doi: 10.1046/j.1472-765X.2002.01200.x.

[12] L. Mannu, R. Comunian, and F.M. Scintu, "Mesophillic lactobacilli in Fiore Sardo cheese: PCR-identification and evolution during cheese ripening", Int. Dairy. J, vol. 10, Oct. 2000, pp. 383-389, doi:10.1016/S0958-6946(00)00074-1.

[13] M. J. Kullen, L. J. Brady, and D. J. O'Sullivan, "Evaluation of using a short region of the recA gene for the rapid and sensitive speciation of dominant bifidobacteria in the human large intestine", FEMS. Microbiol. Lett, vol. 154, Sep. 1997, pp. 377–383, doi: 10.1111/j.1574-6968.1997.tb12670.x.

[14] M.H. Østile, L. Eliassen, A. Florvaag, and S. Skeie, "Phenotypic and PCR-based characterization of the microflora in Norvegia cheese during ripening", Int. J. Food. Microbiol, vol. 94, Aug. 2004, pp. 287-299, doi:10.1016/j.ijfoodmicro.2004.01.012.

[15] O. Kandier, and N. Weiss, Genus Lactobacillus, Bergey's manual of systematic Bacteriology, United Stated of America, 1996, pp: 1208-1234.

[16] P. Chagnaud, K. Machinis, A.L. Coutte, A. Marecat, and A. Mercenier, "Rapid PCR-based procedure to identify lactic acid bacteria:application to six common Lactobacillus species", J.

Microbiol. Meth, vol. 44, Mar 2001, pp. 139-148, doi: 10.1016/S0167-7012(00)00244-X.

[17] P.T. Beresford, N.A. Fitzsimons, L.N. Brennan, and M.T. Cogan, "Recent advances in cheese microbiology", Int. Dairy. J, vol. 11, Jul. 2001, pp. 259-274, doi:10.1016/S0958-6946(01)00056-5.

[18] R. Abdi, M. Sheikh-Zeinoddin, and S. Soleimanian-Zad, "Identification of Lactic Acid Bacteria Isolated from Traditional Lighvan Cheese" Pak. J. Biol. Sci, vol. 9, 2006, pp. 99-103, doi: 10.3923/pjbs.2006.99.103.

[19] R. Osawa, K. Kuroiso, S. Goto, and A. Shimizu, "Isolation of Tannin-Degradation Lactobacilli from Humans and Fermented Foods", Appl. Environ. Microb, vol. 66, Jul. 2000, pp. 3093-3097.

[20] S. Torriani, E.G. Felis, and F. Dellaglio, "Differentiation of Lactobacillus plantarum, L. pentosus and L. paraplantarum by recA Gene Sequence Analysis and Multiple PCR Assay With recA Gene-Derived Primers", Appl. Environ. Microbiol, vol. 67, Aug. 2001, pp. 3450-3454, doi: 10.1128/AEM.67.8.3450-3454.2001.

[21] S. Torriani, F. Clementi, M. Vancanneyt, B. Hoste, F. Dellaglio, and K. Kersters, "Differentiation of Lactobacillus plantarum, L. pentosus and L. paraplantarum Species by RAPD-PCR and AFLP", Syst. Appl. Microbiol, vol. 24, Sep. 2001, pp. 554-560, doi:10.1078/0723-2020-00071.

[22] S.D. Peterson, and R.T. Marshall, "Non starter lactobacilli in Chedar cheese: A review", J. Dairy. Sci, vol. 73, Jun. 1990, pp. 1395-1410, doi:10.3168/jds.S0022-0302(90)78804-2.