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1 Microbial characterization of Iranian traditional Lighvan cheese over manufacturing and ripening
2 via culturing and PCR-DGGE analysis: identification and typing of dominant lactobacilli

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Abstract

This paper reports on the diversity and dynamics of the dominant microbial populations during manufacturing and ripening of Lighvan, a traditional, starter-free Iranian cheese made from raw ewe's and goat's milk, as determined by culturing and PCR-DGGE. Similar dominant populations, composed of *Lactococcus lactis* and *Lactobacillus* spp. strains, were found by both techniques. However, discrepancies regarding the identity of these *Lactobacillus* species were encountered. *Lactobacillus curvatus* and *Lactobacillus sakei* proved to be dominant by PCR-DGGE in milk and 4- and 13 week-old cheese samples; however, *Lactobacillus brevis*, *Lactobacillus plantarum*, *Lactobacillus paraplantarum* and *Lactobacillus paracasei* were the majority cultivable organisms. RAPD typing of lactobacilli isolates showed wide genetic diversity among the different species. Moreover, species and strains evolve over manufacturing and ripening. *L. brevis* and *L. paraplantarum* were dominant in milk but were replaced by *L. plantarum* and *L. paracasei* strains as ripening progressed.

1. Introduction

Lighvan cheese is a semi-hard, starter-free, traditional Iranian cheese from the mountainous area of Lighvan in the Province of Azerbaijan, Iran, made from a mixture of raw ewe's and goat's milk. The manufacture of Lighvan cheese involves coagulation of evening and morning milk with lamb rennet at 28-32 °C (depending on the season). The coagulum is cut into walnut-size pieces that are then transferred to rectangular-shaped bags and piled up for whey drainage. The resulting curd mass is then cut into 25×25×25 cm³ cubes and placed in a 22% brine flow for 6 h. After removal, the curd cubes are kept in a basin for 3-5 days, in which whey drainage continues. During this period the cubes are turned upside down between 9 and 15 times. Finally, they are packed in 10-12% brine. Ripening takes place in deep-natural or man-made caves for 3-4 months at an average temperature of 10-12 °C.

Despite the popularity of Lighvan cheese and its ever increasing consumption figures, its chemical composition has been little studied. Even less is known about the microbial communities involved in its manufacture and ripening; only the dominant bacterial genera of mature cheeses have been reported [1, 2]. Knowledge in this area is of particular interest since the typical sensorial properties of traditional cheeses rely on the complex biochemical processes driven by the indigenous microbiota [3] (as well as on the

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50 animal breeds used to provide the milk, their nutrition, and the cheesemaking practices followed). Further,
51 the Iranian Organization of Standardization now recommends the universal pasteurization of milk for
52 improving the safety of cheeses. Unfortunately, pasteurization denatures milk proteolytic and lipolytic
53 enzymes that might contribute to the characteristics of Lighvan cheese, and can eliminate key
54 microorganisms involved in cheese acidification and/or ripening [4]. Commercial starter cultures therefore
55 become necessary, which could lead to the loss of its ‘genuine’ characteristics [5, 6]. However, starters
56 based on autochthonous microorganisms, which should be in equilibrium with endemic phages [7] and
57 adapted to the milk produced in the manufacturing area, should be able to establish themselves in the
58 cheese matrix better than commercial cultures [8]. They would also help ensure that the particular sensorial
59 properties of this cheese are not lost [9].

60 The microbial characterization of food ecosystems is currently performed using an array of culture-
61 independent molecular techniques [10] (these are faster, more reliable and cheaper than conventional
62 culturing techniques), followed by identification of the microorganisms by biochemical and physiological
63 tests. Denaturing gradient gel electrophoresis (DGGE) can be used to track changes in microbial
64 communities via sequence-specific separation of PCR-amplified fragments [11]. The technique has been
65 used to characterize the microbial diversity of many dairy environments [12-16], and to follow microbial
66 population dynamics over cheese manufacture and ripening [17-21]. In some cases, however, a
67 combination of culturing and molecular techniques is preferred since complementary results are obtained
68 [22, 23]. In addition, culturing allows the isolation, identification and selection of starter and adjunct
69 cultures.

70 This paper reports the basic chemical characteristics of Lighvan cheese during manufacture and
71 ripening, as well as a preliminary inspection of the dominant microbial communities as determined by
72 DGGE. In addition, the *Lactobacillus* species in milk, curd and cheese at different stages of ripening
73 (lactobacilli were the majority species throughout ripening) were identified by phenotypic and molecular
74 methods. The characterization of these lactobacillus strains would be of much help in the design of specific
75 starter cultures for the production of standardized Lighvan cheese.

76
77 **2. Materials and Methods**

2.1. Sampling conditions

Milk, curd and cheeses at 10, 30, 45, 75 and 90 d of ripening from different batches were obtained from three Lighvan cheese producers. Samples were taken according to standard FIL-IDF 50B [24] and transported to the laboratory under refrigerated conditions.

2.2. Chemical analysis

Standard FIL-IDF methods were used to determine basic chemical variables. FIL-FID Standards 21 B [25] and 4 A [26] were followed for examining total solids in milk and cheese respectively. pH was measured according to FIL-FID Standard 104 A [27], and the NaCl and protein content according to FIL-FID Standards 88 A [28] and 20 B [29] respectively.

2.3. DGGE analysis

2.3.1. Extraction of DNA from cheese. Homogenized cheese samples in 2% sodium citrate were used for DNA isolation. DNA extraction was accomplished essentially as described by Ercolini et al. [30], but with the following modification: cheese homogenates were treated with pronase (2.5 mg/ ml) (Sigma Chemical Co., St. Louis, Miss., USA) for 1 h at 37 °C before lysis of the cells.

2.3.2. PCR amplification. Total DNA from cheese samples was used as a template in PCR-amplifications of the V3 region of the bacterial 16S rRNA gene using universal primer F357 (5'-TACGGGAGGCAGCAG-3', to which a 39 bp GC sequence was linked to give rise to GC-F357) and R518 (5'-ATTACCGCGGCTGCTGG-3') [11]. The D1 domain of the 26S rRNA fungal gene was amplified with primers GC-NL1 (5'-GCCATATCAATAAGCGGAGGAAAAG-3') and LS2 (5'-ATTCCCAAACAACCTCGACTC-3') [15].

PCR was performed in 50 µL volumes containing 10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM of each dNTP, 0.2 mM of the primers, 1.5 U of Taq-polymerase (Roche Diagnostics, Barcelona, Spain) and 100 ng of extracted DNA. The conditions established for the amplification of the prokaryotic and eukaryotic sequences were those described by Muyzer et al. [11] and Cocolin et al. [15] respectively.

2.3.3. Electrophoresis conditions. DGGE was performed using a DCode apparatus (Bio-Rad, Richmond, Ca., USA) at 60 °C and employing 8% polyacrylamide gels with a denaturing range of 40-60% for bacteria and 30-50% for fungi. Electrophoresis was conducted at 75 V for 17 h and at 130 V for 4.5 h

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for bacterial and fungal amplifications respectively. Bands were visualized using a UV transilluminator after staining with 0.5 µg/ml ethidium bromide (Sigma).

2.3.4. *Identification of DGGE bands.* DNA bands in the polyacrylamide gels were assigned to species by either comparison with a control ladder corresponding to known strains [17] or, after isolation, by reamplification with the same primers without the GC-clamps followed by sequencing. The sequences obtained were compared with those in the GenBank database using the BLAST program (<http://www.ncbi.nlm.nih.gov/BLAST/>), and with those of the Ribosomal Database Project (<http://rdp.cme.msu.edu/index.jsp>); a species was allocated when identities of >97% were detected [31, 32].

2.4. *Microbial counts*

Cheese samples were emulsified in sterile 2% (w/v) sodium citrate, serially diluted in sterile saline solution, and plated in duplicate on PCA, YGC, M17 and MRS agar plates (Merck) (pH 5.4-5.5). All plates were incubated at 30 °C for 72 h in aerobic conditions.

2.5. *Identification and typing of lactobacilli*

Colonies of all morphologies on the MRS agar plates (MRS is selective for lactobacilli) were chosen at random, purified twice by sub-culturing, and maintained at -20 °C in MRS to which 15% glycerol (w/v) (a cryoprotectant) had been added until analysis.

Total genomic DNA from isolates was prepared as described for the total microbial DNA in cheese, but excluding the pronase treatment. Isolates were grouped by random amplification of polymorphic DNA (RAPD) analysis using primer M13, as reported by Fontana *et al.* [33]. The similarity of the patterns was expressed by the Pearson's product moment correlation coefficient. Clustering was performed by the unweighted pair group method using arithmetic averages (UPGMA). A reproducibility study to determine the minimum percentage of similarity necessary for strain discrimination was performed according to Sánchez *et al.* [34]. The level of similarity obtained established a discrimination threshold below which patterns were considered to belong to different strain. Representative strains with distinct RAPD patterns were identified by partial amplification (using universal primers) and sequencing of the 16S rRNA gene.

Strains of the *Lactobacillus plantarum* group (*L. plantarum*, *L. paraplantarum* and *L. pentosus*) were distinguished by multiplex PCR using species-specific *recA* gene-based primers as described by Torriani *et al.* [35].

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134 **3. Results**135 *3.1. Basic chemical characteristics of Lighvan cheese*

136 Table 1 shows the gross chemical compositions of the milk, curd and cheese at 10, 30, 45, 75, and 90 d
137 of ripening. Although only small standard deviations were returned for most of the variables studied,
138 random differences could be due to the analysis of samples from distinct producers and batches. Generally,
139 cheese total solids increased over ripening as a result of water desorption (water in the cheese moving out
140 to the brine). Consequently, the NaCl content of the cheese increased; indeed, extremely high salt contents
141 were recorded in early ripening (around 20% at day 10). The pH of the curd was much lower than that of
142 the milk, but it recovered slowly over ripening. The high NaCl content and low pH probably determine the
143 changes in microbial populations over ripening.

144 *3.2. Analysis of Lighvan cheese by DGGE*

145 Three samples of Lighvan cheese made by independent producers were initially analyzed by the DGGE
146 after microbial DNA extraction and PCR amplification of the V3 region of the 16S rDNA. The variability
147 in microbial composition, assessed by number of bands and migration position in the DGGE gels, was very
148 low among the analyzed samples. Consequently, one of the samples was chosen as representative of
149 Lighvan cheese and used for the further analysis.

150 Figure 1 summarizes the DGGE profiles for the V3 region of the 16S rRNA gene (bacteria) and those
151 for the D1 domain of the 26S rDNA gene (fungi). Overall, 12 bacterial bands were recorded, corresponding
152 to 11 species (Fig. 1 and Table 2). Each band corresponded to a different species, except for two bands
153 (Fig. 1 A, bands a) that belonged to species of the *Lactobacillus plantarum* group (*L. plantarum*, *L.*
154 *pentosus* or *L. paraplantarum*). In addition, an overall eight fungal bands were obtained that belonged to
155 five different species (Fig. 1B); three of these bands (bands m) belonged to *Debaryomyces hansenii* alone.
156 For the bacterial bands, the highest diversity was obtained in the curd sample (seven different bands) (line 1
157 in Figure 1A). In contrast, the most fungal sequences (six bands) were observed at the end of ripening (lines
158 4 and 5, from 75- and 90 d-old cheese, respectively) (Fig. 1B).

159 The dynamics of the microbial species can be followed in both Figure 1 and Table 2. Some bands
160 increased and others decreased in intensity during ripening. For example, the band corresponding to

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161 *Lactococcus lactis* (band h in Fig. 1A) was very intense in curd samples, after which it became gradually
162 less intense before disappearing in the 90 d-old cheese. Similarly, the band of *Enterococcus*
163 *faecalis/Enterococcus faecium* (band b) was only observed in curd and 30 d-old cheese. The intense band
164 ‘f’ corresponding to *Streptococcus parauberis* abruptly disappeared in sample S4. In contrast, some bands
165 only appeared late in ripening, e.g., those pertaining to *Streptococcus gallolyticus* (band g in Fig. 1A) and
166 *Lactobacillus casei/Lactobacillus paracasei* (band k). This was also observed in the fungal populations.
167 The band corresponding to *Candida zeylanoides* (band l in Fig. 1B) became less intense over ripening,
168 while those corresponding to *D. hansenii* increased in intensity (Fig. 1B).

169 Of particular interest was a bacterial band (band i) belonging to *Escherichia coli*; this was present at all
170 stages of manufacture and ripening at almost the same intensity. An *Enterobacteriaceae*-related band was
171 also identified in the 90 d cheese samples (band j; *Serratia* spp.). Also of note was the presence of several
172 bands of high intensity for some samples that were completely absent for others. The majority of these
173 bands belonged to *Lactobacillus* species, such as *L. plantarum* in sample S4 (band a), *Lactobacillus sakei*
174 present in only sample S5 (band d), and *Lactobacillus curvatus* present only in samples S2 (a 30 d old
175 cheese), S4 and S5 (band e).

176 3.3. Microbial counts

177 Counts of total mesophilic bacteria, lactococci and lactobacilli over manufacturing and ripening were
178 obtained via serial dilution of the samples and plating on appropriate media (Fig. 2). Counts for all were
179 high in the milk (from 2.3×10^6 cfu/ml for lactobacilli to 1.9×10^7 cfu/ml for total mesophilic bacteria). They
180 continued to increase by about two logarithmic (log) units in the curd (up to 8.8×10^8 cfu/g for total viable
181 microorganisms). A sharp reduction (about two log units) was recorded in all counts at four weeks, with
182 figures becoming relatively stable after this date. With the exception of the milk sample, lactococci and
183 lactobacilli were found at similar levels in most samples. On YGC medium, counts for yeasts and moulds
184 were always below the detection limit (<100 cfu/g) (data not shown).

185 3.4. Identification, typing and population dynamics of lactobacilli

186 DGGE and plate enumeration showed lactobacilli to be a typical and distinctive mark of Lighvan
187 cheese; as mentioned, different *Lactobacillus* species were detected by this technique. Therefore, attempts
188 were made to accurately characterise the lactobacilli.

Of the 165 isolates randomly selected from MRS plates for milk and 30 and 90 d-old cheeses analyses, 126 proved to be Gram positive, catalase negative bacilli. All 126 were subjected to RAPD using primer M13 to group strains before identification; 28 different genotypes were obtained with a minimum similarity level of 87% (the value obtained in the reproducibility study) (Fig. 3). The genotypes were numbered (G1-G28) and marked with a letter to indicate their origin (M for milk, Y for the 30 d-old cheese, and R for the 90 d-old cheese). The genotypes separated into two unrelated clusters (C1 and C2) which were subsequently split into 11 groups with a similarity level of 58% (Fig. 3). Representative strains of these different groups were subjected to molecular identification by partial amplification of the 16S rRNA gene, sequencing and comparison of the sequences to those in databases. The 11 groups were assigned to three different species: *Lactobacillus brevis*, *Lactobacillus paracasei* and species of the *L. plantarum* group. Each group represented only one species, but the same species was found in different groups, revealing the high genetic diversity among Lighvan *Lactobacillus* strains. Cluster C1 included all strains of *L. brevis*, and embraced two groups and seven genotypes (21 isolates). Cluster C2 embraced 9 groups and 21 different genotypes, including strains of *L. paracasei* (3 groups, 10 genotypes, 30 isolates) and strains of the *L. plantarum* group (6 groups, 11 genotypes, 75 isolates). The isolates of this last species were all subjected to multiplex PCR for distinguishing among the different species (*L. paraplantarum*, *L. pentosus* and *L. plantarum*). Only *L. paraplantarum* and *L. plantarum* strains were found, representing four of the 11 groups (8 genotypes, 64 isolates) and two of the 11 groups (3 genotypes, 11 isolates) respectively.

The change in the species and genotypes present over manufacturing and ripening was followed by tabulating the frequencies of isolation (Table 3). Most genotypes were detected once during ripening. A few (such as G22, G26 and G18) were detected twice. *Lactobacillus brevis* and *L. paraplantarum* were the majority species in the milk, accounting for 46% and 54% of the isolates respectively. After 30 d of ripening *L. paraplantarum* became the dominant species (86% of the isolates), while in the mature cheeses (90 d) *L. paracasei* predominated (61%) followed by *L. plantarum* (14.3%) and *L. paraplantarum* (14%).

4. Discussion

DGGE has been successfully used to identify and track microbial communities in dairy environments [12-16], including those involved in the manufacture and ripening of traditional cheese made from raw milk

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[17-21]. The length and species-specific heterogeneity of the V3 region of the bacterial 16S rRNA gene, and of the D1 region of the fungal 26S rRNA gene, render them among the best choices for distinguishing bacterial [13, 16, 19, 21] and fungal [15, 17] species respectively. However, limiting factors, such as heterogeneous copies of rDNA operons, annealing mismatches and preferential amplification have been reported [36, 37].

The bacterial and fungal species detected by DGGE in Lighvan cheese have all been reported present in dairy-related environments. The detection of intense bands in some samples but not in others can be attributed to the analysis of cheese samples from different batches. This phenomenon may indicate that *Lactobacillus* species commonly grow to high densities during the ripening of Lighvan cheese (see enumeration results), but the dominant species may be different from batch to batch. The production of two bands by *L. plantarum* and *L. paracasei* strains has been reported before [17]. Further, different copies of the rRNA genes producing distinctive bands by DGGE are found in fungi, as this work shows for *D. hansenii*. It is worth noting that fungal species might be in such low numbers (<100 cfu/g) that enumeration by dilution and plating may not always be possible. However, they can still be tracked by DGGE using specific primers. Thus, PCR-DGGE was considered a convenient technique for a rapid inspection of the diversity and relative abundance of the microbial populations throughout manufacturing and ripening of Lighvan cheese.

Other traditional cheeses have bacterial and fungal population dynamics similar to those found for Lighvan cheese [17, 18]: the bacterial diversity is greater in milk and curd samples, becoming reduced as ripening progresses, while fungal populations increase moderately over ripening [15, 17]. This is probably related to the unfavourable conditions for bacterial growth in cheese (low pH, high salt concentration, presence of organic acids, etc.) compared to the conditions reigning in milk. Even so, these harsh conditions allow the growth of many yeast and mould species.

The high bacterial counts in the milk may be a consequence of storing evening milk at room temperature; this is then mixed with fresh morning milk in the manufacturing process. The microbial differences between samples, which followed no single trend were related to the distinct batches analyzed. Uncontrolled environmental conditions affecting the different batches may also account for some differences, as reported for many other cheese varieties [38-40]. The sharp reduction in the bacterial

populations between the curd and 30 d-old cheese samples might be ascribed to the high salt concentration of the brines (22% w/v for the initial brine and 12% for the ripening brine). *Enterobacteriaceae* species and coliforms could survive the harsh conditions imposed by this salt. Thus, bands corresponding to *E. coli* and *Serratia* spp. were detected even in the 90 d-old cheese and, although they were not further identified, Gram negative organisms were observed among the 165 isolates obtained in MRS. Coliform populations are considered an indicator of faecal contamination, thus the presence of these microorganisms in fully mature Lighvan cheese stresses the need to improve the hygiene associated with its manufacture.

Four *Lactobacillus* species were found among the isolates from the milk and cheese samples: *L. paracasei*, *L. paraplantarum*, *L. brevis*, and *L. plantarum*. These have been reported among the dominant lactobacilli in non-cooked traditional cheeses [34, 38-41]. Further, wide genetic diversity has been reported for cheese-associated lactobacilli [34, 41]. In Lighvan cheese, numerous genotypes were detected in just one sample, and a few were detected twice. This suggests there is rapid succession of species and strains over manufacturing and ripening. Though variations due to the analysis of independent batches may lead to variations in results, *L. brevis* and *L. paraplantarum* strains seem to dominate in milk, but these strains are replaced by *L. plantarum* and *L. paracasei* strains as ripening progresses.

It was not surprising to find enterococci-related amplicons, i.e., bands matching *E. faecalis*/*E. faecium* sequences, or those corresponding to *S. parauberis* and *S. gallolyticus*. Enterococci-like bacteria develop and survive well in dairy products, and have repeatedly been reported in high numbers in products made of raw milk [42]. DGGE bands corresponding to enterococcal or related species have been identified in several cheeses [14, 21, 30].

The culturing results did not agree well with the DGGE results. With DGGE, a prominent band, identified as belonging to *L. curvatus*, was observed in 4 week-old cheese samples (band e, line 2, Fig. 1A). Similarly, intense bands identified as belonging to *L. curvatus* and *L. sakei* species were observed in 13 week-old cheese samples, plus a faint band corresponding to *L. casei*/*L. paracasei*. However, *L. paracasei* strains were dominant among the isolates while *L. curvatus* and *L. sakei* strains were never identified by culturing. Discrepancies between culturing and DGGE results have been reported before [19-21]. In general, DGGE and other molecular techniques do not require organisms to be alive for their sequences to be amplified; DGGE therefore detects the presence of non-viable as well as viable organisms. Moreover,

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273 differential resistance of microbial species to the lytic enzymes used in DNA preparation might account for
274 these differences. The fact that some species are only identified by one of the two methods stresses the
275 importance of combined approaches for fully describing the microbiota of naturally-fermented cheeses.
276 In conclusion, this work provides a first microbiological study of Lighvan cheese by means of
277 culturing and PCR-DGGE analysis, and a detailed identification and typing of the lactobacilli involved in
278 its manufacturing and ripening. The technological characterization of some of these isolates, which is
279 currently in progress, should allow the selection of appropriate strains to be used as adjunct cultures for the
280 standardization and improvement of overall cheese quality and safety.

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Table 1. Basic chemical parameters of the cheese milk and Lighvan cheese samples throughout manufacture and ripening.

Parameter	Stay during manufacture and ripening						
	Milk	Curd	10d	30 d	45 d	72 d	90 d
Total Solids (%)	19.70±2.98	25.23±1.03	31.30±1.72	37.63±1.10	37.86±1.50	38.48±0.91	40.10±2.4
Fat (%)	7.94±0.70	12.50±0.91	7.94±0.70	18.45±0.77	17.66±1.35	19.35±1.56	20.30±2.01
Protein (%)	5.73±0.84	9.70±0.57	5.73±0.84	15.30±0.71	14.90±0.52	16.00±0.69	15.64±0.88
pH	6.58±0.11	4.87±0.21	5.22±0.11	5.30±0.22	5.45±0.28	5.33±0.16	5.02±0.32
NaCl (%)	-	-	14.6±0.13	12.54±0.17	12.88±0.44	13.11±0.28	13.57±0.55
Average S/M (%)	-	-	21.15	20.0	20.6	21.3	22.6

Mean ± standard deviation of three independent measurements.

-, not determined; S/M, salt in moisture

Table 2. Identification of DGGE bands of bacterial and fungal populations from traditional Lighvan cheese and species evolution during manufacture and ripening.

Species (corresponding band/s in Figure 1)	Stay of manufacturing and ripening				
	Curd	30 d	45 d	75 d	90 d
Bacterial populations (Figure 1A)					
<i>Lactobacillus plantarum</i> /	++	+	-	+++	-
<i>Lactobacillus paraplantarum</i> (a)					
<i>Enterococcus faecalis</i> (b)	++	+	+	-	-
<i>Lactobacillus versmoldensis</i> (c)	+	+	+	+	-
<i>Lactobacillus sakei</i> (d)	-	+++	-	++	++
<i>Lactobacillus curvatus</i> (e)	-	+++	+	+++	+++
<i>Streptococcus parauberis</i> (f)	+++	+++	+++	-	-
<i>Streptococcus gallolyticus</i> (g)	+	+	+	-	++
<i>Lactococcus lactis</i> (h)	++++	+++	++	+	-
<i>Escherichia coli</i> (i)	++	++	++	++	++
<i>Serratia</i> spp. (j)	-	-	-	-	++
<i>Lactobacillus casei</i> /					
<i>Lactobacillus paracasei</i> (k)	-	-	-	+	+++
Fungal populations (Figure 1B)					
<i>Candida zeylanoides</i> (a)	++	+++	+	-	-
<i>Debaryomyces hansenii</i> (b, c, d, and h)	+	++	++	+++	+++
<i>Kluyveromyces lactis</i> /					
<i>Kluyveromyces marxianus</i> (e)	+	+++	-	-	-
<i>Candida</i> spp. (f)	-	+++	-	-	-
<i>Rhodotorula mucilaginosa</i> (g)	-	-	-	-	++

+, Relative intensity of bands.

Table 3. Frequency of isolates of the different lactobacilli genotypes and source of isolation during manufacturing and ripening of Lighvan cheese.

Species	Genotype	No. of isolates	Source of isolation		
			Milk (M)	30 d cheese (Y)	90 d cheese (R)
<i>L. brevis</i>	G1	4			4
	G2	4	4		
	G3	4	4		
	G4	3	3		
	G5	2	2		
	G6	2	2		
	G7	2	2		
<i>L. plantarum</i>	G9	3			3
	G20	3			3
	G21	5		2	3
<i>L. paraplantarum</i>	G8	2	2		
	G22	16		16	
	G23	5	5		
	G24	6			6
	G25	5	5		
	G26	23	4	19	
	G27	4	4		
<i>L. paracasei</i>	G28	3	3		
	G10	4			4
	G11	3			3
	G12	2			2
	G13	3			3
	G14	3			3
	G15	2			2
	G16	2			2
	G17	4			4
	G18	4		2	2
	G19	3			3
TOTAL		126	40	39	47

Figure 1

Figure 2

Figure 3

LEGENDS TO THE FIGURES

Figure 1. DGGE profiles of microbial populations from the Iranian Lighvan cheese during manufacturing and ripening. Samples: 1, curd, 2, 3, 4, and 5 cheeses of 30, 45, 75 and 90 d. Panel A: DGGE profiles of the V3 variable region of the bacterial 16S rRNA gene. Key of bands: a, species of the *Lactobacillus plantarum* group; b, *Enterococcus faecalis/Enterococcus faecium*; c, *Lactobacillus versmoldensis*; d, *Lactobacillus sakei*; e, *Lactobacillus curvatus*; f, *Streptococcus parauberis*; g, *Streptococcus gallolyticus*; h, *Lactococcus lactis*; i, *Escherichia coli*; j, *Serratia* spp.; k, *Lactobacillus casei/Lactobacillus paracasei*. Panel B: DGGE profiles of PCR amplicons of the domain D1 of 26S rDNA representing the fungal biodiversity and evolution. Key of bands: l, *Candida zeylanoides*; m, *Debaryomyces hansenii*; n, *Kluyveryomyces lactis/Kluyveromyces marxianus*; p, *Saccharomyces servazzi*; q, *Rhodotorula mucilagenosa*.

Figure 2. Total viable aerobic microorganisms, lactococci and lactobacilli in milk and 30 and 2 d, 4 week, 6 week, 10 week, 13 week old cheeses. Counts were scored on PCA, M17 and MRS, respectively.

Figure 3. Dendogram obtained after RAPD-PCR analysis of mesophilic lactobacilli isolated through fermentation process of Lighvan cheese. RAPD pattern obtained after amplification with M13 primer were grouped by means of the Pearson product moment correlation coefficient and UPGMA cluster analysis with MVSP software (Kovach Computing Services, Anglesey, UK).