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Abstract: The microbial populations of three batches of Lighvan cheese and one of Koozeh cheese, the most important traditional Iranian cheeses, were tracked throughout manufacture and ripening by denaturing gradient gel electrophoresis (DGGE), a culture-independent microbial technique. In addition, the majority components of the lactic acid bacteria species were identified by molecular methods following their culturing on selective media. The dominant amplicons in all four cheese batches were found to belong to the species *Streptococcus parauberis* and *Lactococcus lactis*. An amplicon corresponding to *Escherichia coli* was also identified in all three Lighvan batches, and another matching that of *Streptococcus thermophilus* was found in one batch of Lighvan cheese (at all monitoring times) and in the single batch of Koozeh. In contrast, *Enterococcus* spp. was the dominant isolate at all times; most of the isolates from both cheese varieties were assigned to *Enterococcus faecium*. This discrepancy suggests that, while contributing to the DNA pool, the dominant populations are in a viable but not cultivatable state during ripening. The results of this work further reinforce the idea that culture-dependent and culture-independent techniques provide complementary results, ultimately affording a better description of cheese ecosystems.

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Dr. Tamime has a recognised reputation in dairy microbiology and the microbiology of traditional products. In addition, his expertise in lactic acid bacteria would be appreciated for the reviewing.

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Dr. A. Thierry
Editor-in-Chief of *Dairy Science and Technology*
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Dear Dr. Thierry,

Attached, please, find a copy of an original manuscript by Edalatian et al. 2011, entitled "Microbial analysis of the traditional Iranian cheeses Lighvan and Koozeh by culturing and culture-independent techniques", which is intended to be published after the mandatory reviewing process in *Dairy Science and Technology* after its convenient reviewing.

This work has been conducted as a collaboration between Iranian and Spanish research groups, which included a training stay of one Iranian PhD student in our laboratory for six months. In addition to a contribution to the microbiology of traditional cheeses, comparing culturing and culture-independent microbial methods allowed to a better description of the microbial cheese ecosystem. Therefore, we think the report may be of interest for dairy microbiology and food technologists, usual readers of the *Dairy Science and Technology* journal.

The results have not been published before and have not been submitted to other journal.

Thank you very many in advance for your consideration, and looking forward to hear soon from you.

Sincerely yours,

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1 Microbial analysis of the traditional Iranian cheeses Lighvan and Koozeh by
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24

25 **Abstract**

26 The microbial populations of three batches of Lighvan cheese and one of
27 Koozeh cheese, the most important traditional Iranian cheeses, were tracked
28 throughout manufacture and ripening by denaturing gradient gel electrophoresis
29 (DGGE), a culture-independent microbial technique. In addition, the majority
30 components of the lactic acid bacteria species were identified by molecular
31 methods following their culturing on selective media. The dominant amplicons in
32 all four cheese batches were found to belong to the species *Streptococcus*
33 *parauberis* and *Lactococcus lactis*. An amplicon corresponding to *Escherichia*
34 *coli* was also identified in all three Lighvan batches, and another matching that of
35 *Streptococcus thermophilus* was found in one batch of Lighvan cheese (at all
36 monitoring times) and in the single batch of Koozeh. In contrast, *Enterococcus*
37 spp. was the dominant isolate at all times; most of the isolates from both cheese
38 varieties were assigned to *Enterococcus faecium*. This discrepancy suggests that,
39 while contributing to the DNA pool, the dominant populations are in a viable but
40 not cultivatable state during ripening. The results of this work further reinforce the
41 idea that culture-dependent and culture-independent techniques provide
42 complementary results, ultimately affording a better description of cheese
43 ecosystems.

44

45 **Key words:** traditional cheeses; lactic acid bacteria; starters; adjunct cultures;
46 DGGE

47

48 **1. Introduction**

49 Lighvan and Koozeh are among the best known and most appreciated of all
50 traditional Iranian cheeses. They are manufactured in the neighboring
51 northwestern provinces of East and West Azerbaijan, respectively. Both are made
52 from raw ewe's milk or a mixture of ewe's and goat's milk following ancient
53 cheese-making technologies; no commercial starters are involved. Fig. 1 shows
54 the main steps in their production. Both have their own tastes and flavor profiles
55 and are enjoying increasing popularity.

56 Starter-free cheeses made from raw milk -such as Lighvan and Koozeh- rely
57 for their acidification and ripening on the action of their indigenous lactic acid
58 bacteria (LAB) [37]. However, for such traditional cheeses to be competitive in
59 national and international markets, product standardization is necessary and food
60 safety must be ensured. This requires the identification, characterization and
61 typing of the key microorganisms which grow in them, and the selection of those
62 appropriate for use as specific starters and adjunct cultures [28]. The use of such
63 cultures would ensure fermentations can be reliably reproduced while preserving
64 the typical, traditional bouquet of these cheeses [34]. The Lighvan and Koozeh
65 ecosystems may also harbor LAB strains with unique flavor-forming capabilities
66 that might be advantageous in different areas of the dairy industry [3] or in the
67 production of new, broad-range antimicrobial agents [4].

68 Rapid microbial inspections of food fermentations can now be performed
69 using an array of culture-independent molecular methods [8, 19]. These
70 complement classic culturing techniques, providing a more complete picture of

71 these products' microbial composition and dynamics. The polymerase chain
72 reaction (PCR) coupled with denaturing gradient gel electrophoresis (DGGE) -
73 PCR-DGGE- is one such culture-independent molecular method that has already
74 been used to characterize the microbial populations of dairy products [7, 12, 16,
75 23]. In fact, such characterization has been performed over the manufacture and
76 ripening of a number of traditional cheeses [9, 14, 16, 32].

77 Few microbial studies have been made on traditional Iranian cheeses.
78 However, pioneering work has identified the dominant LAB species in ripened
79 Lighvan cheese [1, 5], as well as the cultivatable lactobacilli that appear during
80 manufacture and ripening [21]. To our knowledge, studies on the microbiota of
81 Koozeh cheese have never been attempted. In this study, PCR-DGGE was used to
82 type the majority microbial populations during the manufacture and ripening of
83 different batches of Lighvan and Koozeh cheeses. In addition, a series of
84 cultivatable LAB strains were isolated from them and subjected to molecular
85 identification and typing. This allowed the identification of the dominant
86 cultivatable LAB species in these cheese ecosystems, plus the assessment of their
87 diversity.

88

89

90 **2. Materials and Methods**

91

92 *2.1. Sampling*

93 In the summer of 2009, three batches of Lighvan and one batch of Koozeh
94 cheeses made by different producers were sampled over manufacture following
95 the traditional technology (Fig. 1) and ripening, i.e., as cheese milk, curd, cheese
96 at 3, 7, 15, 30, 60, and 90 days after manufacture. Samples were then transferred
97 to the laboratory under refrigerated conditions, and analyzed within 6 h of arrival.

98

99 2.2. *DGGE analysis of Lighvan and Koozeh cheeses*

100

101 2.2.1. Extraction of total microbial DNA

102 Milk, curd and cheese samples at 3, 7, 15, 30, and 60 days were homogenized
103 in 2% sodium citrate and the homogenates used for the isolation of total microbial
104 DNA employing the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany)
105 following the manufacturer's instructions. DNA quantity and quality was
106 measured by absorption at 260 and 280 nm using a Nanodrop spectrophotometer
107 (Thermo Scientific, Wilmington, DE, USA).

108

109 2.2.2. PCR amplification of 16S rRNA sequences

110 Purified DNA was used as a template in PCR amplifications of the V3 region
111 of the bacterial 16S rRNA gene using the universal primers F357-GC clamp (5'-
112 TACGGGAGGCAGCAG-3', to which a 39 bp GC sequence was linked) and
113 R518 (5'-ATTACCGCGGCTGCTGG-3'), as reported by Muyzer et al. [25]. The
114 amplification of the D1 domain of the 26S rRNA gene of fungi was accomplished
115 using the primers NL1-GC (5'-GCCATATCAATAAGCGGAGGAAAG-3', with

116 a 39 bp GC clamp) and LS2 (5'-ATTCCCAAACAACCTCGACTC-3'), as reported
117 by Cocolin et al. [7]. PCR was performed in 50 μ L reaction volumes containing
118 10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM of each dNTP, 0.2 mM of
119 the primers, 5 U of Taq-polymerase and 100 ng of DNA.

120

121 2.2.3. Electrophoretic conditions

122 DGGE was performed using a DCode apparatus (Bio-Rad) at 60°C and
123 employing 8% polyacrylamide gels with a denaturing range of 40-60% for
124 bacteria and 30-50% for fungi. Electrophoresis was performed at 75 V for 16 h
125 and 130 V for 4.5 h for bacterial and fungal amplifications respectively. Bands
126 were visualized under UV light after staining with ethidium bromide (0.5 μ g ml⁻¹),
127 and photographed.

128

129 2.2.4. Identification of DGGE bands

130 DNA bands in the polyacrylamide gels were assigned to species by
131 comparison with a control ladder of known strains [16]. DNA was isolated by
132 elution from the bands that did not migrate to the positions of the controls, re-
133 amplified with the same primer pair without the GC-clamp, sequenced, and the
134 sequences compared as above.

135

136 2.3. Microbial analysis

137 Milk samples were diluted in 0.1% sterile peptone water. Twenty five grams
138 of curd and cheese samples at day 30 (fresh cheese) and at day 90 (ripened

139 cheese) were homogenized in 225 ml of a sterile sodium citrate solution (2% w/v)
140 using a Stomacher 400 (Seward, Worthing, UK). Dilutions of milk, curd and
141 cheese samples were then plated, with the isolation and enumeration of
142 lactobacilli, lactococci, enterococci, and leuconostoc performed in duplicate on
143 agarified plates of MRS (Merck, Darmstad, Germany), M17 (Scharlab, Barcelona,
144 Spain), KAA (Oxoid, Basingstoke-Hampshire, UK) and MRS agar plus
145 vancomycin (20 µg/ml) respectively. Plates were incubated aerobically in a
146 GasPack EZ system (BD, Franklin Lakes, NJ, USA) at either 30°C (*Lactococcus*,
147 *Leuconostoc*), 37°C (*Lactobacillus*) or 42°C (*Enterococcus*) for 24-72 h. Four to
148 five representative colonies (according to shape, size and color) were selected
149 randomly, purified two or three times on the same media, and examined for Gram
150 staining, catalase production and morphology. All Gram-positive, catalase-
151 negative isolates were selected for further identification and stored frozen in MRS
152 broth containing 20% glycerol. In total, 130 isolates (82 from Lighvan and 48
153 from Koozeh) were identified by molecular methods.

154

155 2.4. Molecular identification of LAB species

156

157 2.4.1. DNA extraction

158 For DNA extraction, cultures stored at -80°C were recovered. Single, isolated
159 colonies were then suspended in 50 µl of molecular grade water (Sigma-Aldrich,
160 St. Louis, MO, USA), heated at 98°C for 10 min in a thermocycler (Bio-Rad,
161 Richmond, CA, USA), and centrifuged for 5 min at 16,000 rpm. For some of the

162 isolates, cell extracts were obtained with glass beads in a Minibead Beater
163 apparatus (Biospec Products, Bartlesville, OK, USA) and centrifuged as above.
164 Cell-free extract supernatants were used as DNA templates for the amplification
165 of a major part of the 16S rRNA gene by PCR.

166

167 2.4.2. Amplification of 16S rRNA genes

168 The primers used for the amplification of the 16S rRNA genes were 27FYM
169 (5'- AGAGTTTGATYMTGGCTCAG- 3') and 1492 R (5'-
170 GGTTACCTTGTTACGACTT- 3'), based on the conserved regions of the 16S
171 rRNA gene. PCR was performed in 50 µl reaction vessels containing 10 mM Tris-
172 HCl, 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM of each dNTP, 0.2 mM of the primers,
173 1.5 U of Taq-polymerase (Amplicon, Skovlunde, Denmark), and 2 µl of the cell-
174 free extracts.

175

176 2.4.3. Amplified ribosomal DNA restriction analysis

177 For amplified ribosomal DNA restriction analysis (ARDRA), amplicons were
178 subjected to digestion with the *Hae*III and *Hha*I restriction enzymes (Invitrogen
179 Ltd., Paisley, UK) and electrophoresed in 1.5% agarose gels at 75 V for 90 min.
180 All gels were stained with ethidium bromide (0.5 µg ml⁻¹), visualized under UV
181 light, and photographed.

182

183 2.4.4. Sequencing and sequence comparison

184 Representative amplicons of the different ARDRA profiles were sequenced by
185 cycle extension in an ABI 373 DNA sequencer (Applied Biosystems, Foster City,
186 Ca., USA) using primer 27FYM. On average, 850 bp were obtained per sequence,
187 which were then compared with sequences in the GenBank database using the
188 BLAST program [6] and with those held by the RDP database [35]. Sequences
189 showing 98% similarity or higher were deemed to belong to the same species [29,
190 36].

191

192 *2.5. Typing of isolates*

193 All isolates were grouped by repetitive extragenic palindromic PCR (REP-
194 PCR) typing using primer BoxA2R (5'- ACGTGGTTTGAAGAGATTTTCG-3')
195 and employing the amplification conditions of Koeuth et al. [22]. PCR products
196 were then electrophoresed and visualized as above. Pattern similarity was
197 expressed via the Simple Matching coefficient, and patterns clustered using the
198 unweighted pair group method using arithmetic averages (UPGMA).

199

200

201 **3. Results**

202

203 *3.1. Microbial dynamics of Lighvan and Koozeh cheeses, as determined by DGGE*

204 Three batches of Lighvan cheese manufactured by independent producers,
205 plus one batch of Koozeh cheese, were analyzed by PCR-DGGE over
206 manufacture and ripening (Figs. 2 and 3, respectively; Online Resource Table 1)

207 using universal primers to track both bacterial and eukaryotic populations. For the
208 Lighvan cheeses, samples of milk, curd and cheeses at days 3, 7, 15, 30, and 60
209 after manufacture were analyzed, while for Koozeh, cheeses at days 3, 7, 15, 30,
210 and 60 were sampled. The number of bacterial DGGE bands obtained for the
211 different samples ranged between four (milk for Lighvan batch 2; Fig. 2B, line 1)
212 and eleven (60 day-old Lighvan batch 1; Fig. 2A, line 7); all were identified at the
213 species level. This large number of bacterial bands contrasts with the small
214 number of eukaryotic populations; only two bands were observed in a 60 day-old
215 sample of Lighvan cheese (data not shown), and between two and four bands in
216 samples of Koozeh cheese over the entire experimental period (Fig. 3B).
217 Eukaryotic bands were mostly identified at the genus level. In total, 25 bacterial
218 (20) and eukaryotic (5) bands were identified by sequencing and sequence
219 comparison.

220 Similarities and differences in the DGGE profiles were noted between the two
221 cheeses, and between the distinct batches of Lighvan. The predominant bands for
222 all cheeses over manufacture and ripening corresponded to the species
223 *Streptococcus parauberis* (band d) and *Lactococcus lactis* (band g). In Lighvan, a
224 band of variable intensity corresponding to *Lactococcus garvieae* (band a) was
225 observed in most cheese samples, as was a band for *Escherichia coli* (band h).
226 *Lactococcus raffinolactis* (band 1) and *Enterococcus faecium* (band e) were also
227 present in two batches of Lighvan at most times. A band with a sequence
228 matching that of *Streptococcus thermophilus* (band 3) was observed in batch 1 of
229 Lighvan cheese at all times, and in the milk and curd samples respectively of the

other two batches (Fig. 2). Bands identified occasionally included *Lactobacillus*
plantarum and *Lactobacillus paracasei* in batch 1 (Online Resource Table 1),
Staphylococcus haemolyticus in the milk samples of batches 2 and 3,
Lactobacillus salivarius in the milk samples of batch 3, and *Macrococcus*
caseolyticus in all samples of batch 3 cheese. DGGE profiles within a single
 Lighvan cheese batch were almost identical over ripening. Greater changes were
 observed, however, for the DGGE profiles of the Koozeh samples (Fig. 3). The
 DGGE profiles of this cheese were dominated by *S. parauberis* (band d), followed
 by *L. lactis* which was present in all samples (band g). However, *L. raffinolactis*
 was present only in 3 and 7 day-old cheese samples. *Streptococcus uberis* (band
 2) was identified in day 3, 7, and 15 samples, *Carnobacterium maltomaricum*
 (band 3) was identified in day 15, *Lactobacillus curvatus* (band 4) in day 30 and
 day 60 samples, and *S. thermophilus* (band 6) was seen in all samples except that
 for day 15. Surprisingly, two bands present in most of the samples were identified
 as *Celerinatantimonas diazaotrophica* (band 5) and *Vibrio tapetis* (band 7);
 microorganisms not usually found in cheese. Bands corresponding to mould and
 yeast populations were obtained in three of the five Koozeh samples. One of the
 eukaryotic bands was related to the ascomycete *Warcupia* spp. (band 8) (Fig. 3)
 and two bands each were identified as belonging to *Debaryomyces hansenii* (band
 9) and *Penicillium* spp. (band 10) (Fig. 3).

250

3.2. Identification and typing of LAB species from Lighvan and Koozeh by
 culturing

253 To characterize the LAB populations of Lighvan and Koozeh over
254 manufacture and ripening, 130 colonies (82 from Lighvan and 48 from Koozeh)
255 grown on enumeration media M17 (48), MRS (58), and KAA (24) were purified,
256 subcultured in the same media, and stored at -80°C until identification. DNA from
257 the cultures was isolated by either heating or by glass bead treatment and used as
258 a template to amplify a major part of the 16S rRNA gene by PCR employing the
259 universal bacterial primers 27F and 1492R. Amplicons were all subjected to
260 ARDRA analysis after treatment with the restriction enzymes *Hae*III and *Hha*I.
261 Eleven different patterns were obtained with either enzyme (Online Resource Fig.
262 1). Representative amplicons of the different profiles were then sequenced and the
263 results compared against those in the GenBank and RDP databases. Table 1 shows
264 the molecular identification results. Nine different bacterial species were
265 encountered in Lighvan and eight in Koozeh during ripening. *Enterococcus* spp.,
266 including *E. faecium*, *E. faecalis*, *E. durans* and other species, made up the major
267 part of the cultures. Of these, *E. faecium* (74 isolates) was dominant in both
268 Lighvan and Koozeh cheese at all times, followed by *E. faecalis* (16 isolates).
269 Although in small numbers, *L. plantarum* (21 isolates) and *Lactobacillus brevis* (6
270 isolates) were occasionally recovered from both cheeses (Table 1). These results
271 contrasted with those obtained in DGGE analysis since the species producing the
272 majority bands were almost absent in the cultures (only four *L. lactis* isolates from
273 Lighvan cheese milk and curd were obtained, while *S. parauberis* isolates were
274 never recovered).

Isolates of the majority species *Enterococcus* spp. and *L. plantarum* were all subjected to REP-PCR typing to evaluate intra-species diversity. Fig. 4 shows the profiles obtained with the 38 *E. faecium* isolates from Lighvan cheese, plus the similarity dendrogram for the different typing patterns clustered by the UPGMA method and using the Simple Matching coefficient. Given the reproducibility of the assay (around 90%; Online Resource Fig. 2), isolates sharing a percentage similarity of >88% (an arbitrary figure) were considered to be the same strain (Fig. 4). Twenty four different profiles were considered to represent different strains. Twenty four different stains were also found among the 36 *E. faecium* isolates from Koozeh cheese (Online Resource Fig. 3). Wide genetic diversity was also detected among the *L. plantarum*, *E. faecalis*, and *E. casseliflavus* isolates (data not shown).

4. Discussion

Few microbial studies of Lighvan cheese have been undertaken [1, 21], and to our knowledge this is the first microbial description of Koozeh cheese. A similar number of species was detected by the culturing and culture-independent methods. Eight and nine different species were identified among the cultured isolates from Koozeh and Lighvan respectively, and 4-11 bands corresponding to an equal number of species were obtained in DGGE analyses. However, the different methods showed discrepancies in terms of the microbial populations

297 identified; they therefore provided complementary results [11, 16, 31, 32]
 298 allowing a better description of these cheese ecosystems to be made.

299 The bacterial and fungal population dynamics recorded by DGGE for the two
 300 cheeses were similar to those reported for other traditional cheeses [16, 33];
 301 bacterial diversity was usually greater in the milk and curd samples with most
 302 species failing to grow in the cheese. Two to four high intensity bands were
 303 observed for the different batches of the two cheeses at all times, which were
 304 accompanied by up to nine bands of lower intensity. The intensity of an individual
 305 DGGE band is assumed to be a semi-quantitative measure of the corresponding
 306 microbe's abundance in the sample [25]. In Lighvan and Koozeh, DGGE
 307 identified the dominant populations as belonging to *S. parauberis* and *L. lactis*
 308 species, with contributions at certain times from *S. thermophilus*, *L. curvatus* and
 309 *C. diazotrophica*. *L. garvieae*, *L. raffinolactis* and *E. coli* were found in most
 310 batches in subdominant numbers. Though *Enterococcus* spp. bands were found in
 311 several batches, as a majority were only encountered in Lighvan cheese batch 1
 312 (Fig. 2A). Thus, it was surprising to discover that species of this genus accounted
 313 for a large proportion of the cultured microorganisms (73.8% of the isolates;
 314 Table 1). These results, however, are not unusual, as DGGE bands corresponding
 315 to enterococcal species have been reported in some cheese types [9, 13, 26] and
 316 not in others [14, 26, 32]. Of note in the sampled cheeses is the presence of bands
 317 related to *S. thermophilus* in most batches; these Iranian cheeses might therefore
 318 be considered a good source of new strains of this important cheese starter. DGGE
 319 bands of *S. thermophilus* have also recently been reported in a traditional Spanish

320 starter-free cheese made from raw cow's milk [2]. The identified strains of *S.*
321 *thermophilus* in the latter cheese have been isolated and are currently being
322 characterized and compared with industrial starter strains (unpublished).
323 Similarly, *L. garvieae*, a lactic acid bacterium similar to *L. lactis* [15], has been
324 identified by culturing and molecular methods in other cheese types [2].

325 The fact that cultivatable strains belonging to the populations of the most
326 prominent bands (*S. parauberis* and *L. lactis*) were not readily recovered on the
327 enumeration plates strongly suggests that these microbial species are in a non-
328 cultivatable state in Lighvan and Koozeh. Similar results have been reported
329 elsewhere for other traditional cheeses [14, 32]. However, the DGGE results
330 indicate that these two populations reach high densities at the beginning of the
331 manufacturing process; certainly, they are present in the milk. *S. parauberis* has
332 been associated with subclinical and clinical mastitis [30]; therefore, its presence
333 in cheese is not desirable. In contrast, *L. lactis* is the typical LAB species of
334 cheese. It enjoys 'generally regarded as safe' (GRAS) status and its enzymes
335 contribute towards the typical taste and aroma profiles of cheese [24, 28]. Specific
336 starters for these Iranian cheeses should therefore include strains of this species.
337 Isolation of such *L. lactis* strains would have to be undertaken at the beginning of
338 cheese manufacture (curdling of the milk, acidification, whey drainage, etc.).

339 Enterococci have been repeatedly reported to form the majority populations in
340 artisanal, traditional cheeses made from raw milk (for a review see [18]). The high
341 enterococci counts in Lighvan and Koozeh agree well with the low pH of these
342 cheeses (average 4.64%) and the high concentration of salt present during

343 ripening (up to 5.27% at day 90). Under such harsh conditions, *Enterococcus* spp.
344 may thrive better than other LAB populations. Though the presence of high
345 numbers of enterococci in foods is controversial [27], strains of some species have
346 been proposed as starters or adjunct cultures for several cheese types [18]. A high
347 intraspecies diversity was found among the enterococci isolates using REP-PCR,
348 which suggests a high subsequent phenotypic diversity (Fig. 4). Moreover, several
349 strains have been shown to produce bacteriocins such as enterocin A, B, P, and X,
350 or a combination of these (unpublished). Certainly, enterococci strains play
351 pivotal roles in the production of aroma compounds [11, 17, 18]. However, for
352 their safe use in food systems, candidate strains would have to be subjected to
353 complete characterization, guaranteeing the absence of recognized virulence
354 factors and atypical, potentially transferable antibiotic resistance elements [17,
355 27].

356 *C. diazotrophica* and *V. tapetis* are both recently reported marine bacteria,
357 which may well come from the salt added to the cheeses. The occasional
358 development of these pathogenic microorganisms and the recurrent presence of
359 opportunistic populations (such as those of *Enterococcus* spp., *S. parauberis*)
360 argue for a need of improvement of the safety conditions of these two traditional
361 cheeses.

362

363

364 **5. Conclusions**

365 This work contributes to the microbial characterization of the most important
366 traditional Iranian cheeses, Lighvan and Koozeh. The fact that some microbial
367 populations were detected by one identification method only stresses the
368 importance of combined approaches for fully describing the microbiota of
369 naturally fermented cheeses. The results of these studies may be useful for the
370 selection of already-available commercial starters for the industrial-scale
371 manufacture of Lighvan and Koozeh cheese using pasteurized milk, or for the
372 selection of strains of LAB species and the design of specific, reliable starters to
373 be used in traditional manufacture. These results may also provide the basis for
374 the future award of protected designation of origin (PDO) status or an equivalent
375 quality label for these cheeses.

376

377

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385

386

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Table 1.- Species and numbers of majority cultured microorganisms identified through manufacturing and ripening of the traditional Iranian cheeses Lighvan and Koozeh.

Species	Stage of manufacture				Total
	Milk	Curd	30 days	90 days	
Lighvan cheese					
<i>Lactococcus lactis</i> subsp. <i>lactis</i>	3	1	-	-	4
<i>Lactobacillus plantarum</i>	3	8	9	-	20
<i>Lactobacillus brevis</i>	2	-	-	3	5
<i>Enterococcus faecium</i>	3	11	11	13	38
<i>Enterococcus faecalis</i>	-	2	-	9	11
<i>Enterococcus durans</i>	1	-	-	-	1
<i>Enterococcus casseliflavus</i>	-	-	1	-	1
<i>Enterococcus italicus</i>	1	-	-	-	1
<i>Micrococcus luteus</i>	-	-	-	1	1
Total Lighvan	13	22	21	26	82
Koozeh cheese					
<i>Enterococcus faecium</i>			7	29	36
<i>Enterococcus faecalis</i>			-	5	5
<i>Enterococcus durans</i>			-	1	1
<i>Enterococcus casseliflavus</i>			-	2	2
<i>Lactobacillus plantarum</i>			1	-	1
<i>Lactobacillus brevis</i>			1	-	1
<i>Staphylococcus haemolyticus</i>			-	1	1
<i>Aerococcus viridans</i>			-	1	1
Total Koozeh			9	39	48
TOTAL	13	22	30	65	130

Fig. 1

Fig.2

Fig. 3

Figure

Fig. 4

FIGURE CAPTIONS

Figure 1.- Diagram and flow chart of manufacturing and ripening stages of traditional, Iranian raw milk cheeses Lighvan (A) and Koozeh (B).

Figure 2.- Bacterial dynamics as shown by the DGGE profiles of the V3 variable region of the bacterial 16S rRNA gene in three independent batches of Lighvan cheese (panel A, B, and C, respectively) throughout manufacturing and ripening. Samples: milk, curd, and cheeses at day 3, 7, 15, 30, and 60 after manufacture. Ma and Mb, DGGE markers used as a control and composed of amplicons of isolated strains, as follows: a, *Lactococcus garvieae*; b, *Lactobacillus plantarum*; c, *Leuconostoc mesenteroides*; d, *Streptococcus parauberis*; e, *Enterococcus faecium*; f, *Enterococcus faecalis*; g, *Lactococcus lactis*; h, *Escherichia coli*, and i, *Lactobacillus paracasei*. Bands identified by sequencing are coded with a letter if corresponding to species on the markers and with a number for other species, as follows: 1, *Lactococcus raffinolactis*; 2, *Lactococcus plantarum*; 3, *Streptococcus thermophilus* (two bands); 4, *Staphylococcus haemolyticus*; 5, *Lactobacillus salivarius*; 6, *Macroccoccus caseolyticus*.

Figure 3.- Microbial dynamics in Koozeh cheese during manufacturing and ripening as judged from DGGE profiles of the V3 variable region of the bacterial 16S rRNA gene (panel A) and the D1 domain of the 26S rRNA gene of fungi (panel B) Samples: cheese at 3, 7, 15, 30 and 60 day after manufacture. Ma and Mb as in Figure 1 (except for absence of the *Lactobacillus plantarum* amplicon; band b). Sequenced bands are denoted by a letter code if corresponding to species on the markers or by a number if they were identified by

reamplification, sequencing and sequence comparison, as follows: 1, *Lactococcus raffinolactis*; 2, *Streptococcus uberis*; 3, *Carnobacterium maltomaricum*, 4, *Lactobacillus curvatus*; 5, *Celerinatantimonas diazotrophica*; 6, *Streptococcus thermophilus*; 7, *Vibrio tapetis*; 8, *Warcupia* spp.; 9, *Debaryomyces hansenii* (two bands); and 10, *Penicillium* spp. (two bands).

Figure 4. Typing REP-PCR profiles obtained with primer BoxA2R among the 38 *Enterococcus faecium* isolates from Lighvan cheese. Below, dendrogram of similarity of the different typing patterns clustered by the UPGMA method using the Simple Matching coefficient. M, molecular weight marker GeneRuler™ (Fermentas, St. Leon-Rot, Germany). The broken line denotes the arbitrary percentage of similarity (88%) used to consider isolates as different strain; this percentage of similarity was lower than the assay reproducibility (90%; Supplementary Figure 2).

Supplementary Table 1.- Microbial dynamics as determined by identification of DGGE bands through manufacture and ripening stages of the traditional Iranian cheeses Lighvan and Koozeh.

Species (band code in figures)	Stage of manufacture or ripening time						
	Milk	Curd	3 d	7 d	15 d	30 d	60 d
Lighvan cheese (batch 1, Figure 2A)							
<i>Lactococcus garvieae</i> (a)	-	(+)	+	+	+	+	+
<i>Lactobacillus plantarum</i> (b)	+	++	-	-	-	-	-
<i>Lactococcus raffinolactis</i> (1)	-	+	+	+	+	+	(+)
<i>Lactococcus plantarum</i> (2)	-	-	-	-	+	+	+
<i>Streptococcus parauberis</i> (d)	++	++++	+++	+++	+++	+++	++
<i>Enterococcus faecium</i> (e)	-	-	+	+	+	++	++
<i>Lactococcus lactis</i> (g)	+	+	+++	+++	+++	++	++
<i>Streptococcus thermophilus</i> (3)	-	+	+++	+++	+++	+++	+++
<i>Escherichia coli</i> (h)	-	-	+	+	+	(+)	(+)
<i>Lactobacillus paracasei</i> (i)	-	-	-	-	-	(+)	+
Lighvan cheese (batch 2, Figure 2B)							
<i>L. garvieae</i> (a)	-	++	++	++	++	++	++
<i>L. raffinolactis</i> (1)	-	++	++	++	++	+	+
<i>Staphylococcus haemolyticus</i> (4)	++	-	-	-	-	-	-
<i>S. parauberis</i> (d)	++	+++	+++	+++	+++	+++	++
<i>L. lactis</i> (g)	+	+	+++	+++	+++	++	++
<i>S. thermophilus</i> (3)	(+)	-	-	-	-	-	-
<i>E. coli</i> (h)	-	-	(+)	(+)	+	(+)	(+)
Lighvan cheese (batch 3, Figure 2C)							
<i>L. garvieae</i> (a)	-	-	++	++	++	++	++
<i>Lb. plantarum</i> (b)	(+)	(+)	-	-	-	-	-
<i>L. raffinolactis</i> (c)	(+)	+	(+)	(+)	(+)	(+)	(+)
<i>Lactobacillus salivarius</i> (5)	+	-	-	-	--	-	-
<i>Enterococcus faecium</i> (e)	+	-	+	(+)	+	+	-
<i>Macroccoccus caseolyticus</i> (6)	-	-	+	(+)	++	++	(+)
<i>S. haemolyticus</i> (4)	+	-	-	-	-	-	-
<i>M. caseolyticus</i> (6)	-	-	+	(+)	+	+	(+)
<i>S. parauberis</i> (d)	++	+++	++	++	++	++	+
<i>Enterococcus faecalis</i> (f)	-	-	+	-	+	+	+
<i>L. lactis</i> (g)	+	+	+++	+++	+++	++	++
<i>S. thermophilus</i> (3)	-	+	-	-	-	-	-
<i>E. coli</i> (h)	-	-	+	+	+	-	+
Koozeh cheese (batch 1, Figure 3)							
<i>L. garvieae</i> (a)			-	-	+	-	(+)
<i>L. raffinolactis</i> (1)			++	+	-	-	-
<i>Streptococcus uberis</i> (2)			+	+	++	-	-
<i>Carnobacterium maltomaricum</i> (3)			-	-	+++	-	-
<i>Lactobacillus curvatus</i> (4)			-	-	-	++	+++
<i>S. parauberis</i> (d)			+++	+++	++++	+++	+++
<i>Celerinatantimonas diazotrophica</i> (5)			(+)	+	-	++	++
<i>L. lactis</i> (g)			++	++	++	++	++
<i>S. thermophilus</i> (6)			++	++	-	++	++
<i>Vibrio tapetis</i> (7)			(+)	+	-	+	+
<i>Warcupia</i> spp. (8)			++	+	-	-	+
<i>Debaryomyces hansenii</i> (9)			++	+	-	-	++
<i>Penicillium</i> spp. (10)			++	-	-	-	-

Number of crosses indicates the relative intensity of bands; the symbol (+) indicates faint bands.

Online Resource Fig. 1. Partial amplified ribosomal DNA restriction analysis (ARDRA) profiles of 11 colonies from Lighvan and Koozeh cheeses from different manufacturing and ripening stages. The 16S rRNA gene was amplified using primers 27F and 1492R and digested with the restriction enzymes *Hae*III (A) and *Hha*I (B). M, molecular weight marker GeneRuler™. After partial amplification, sequencing and sequence comparison of 16S rRNA genes, the profiles were shown to correspond to the following species: 1, *Enterococcus faecium*; 2, *Lactobacillus plantarum*; 3, *Lactobacillus brevis*; 4, *Lactococcus lactis* subsp. *lactis*; 5, *Enterococcus faecalis*; 6, *Enterococcus durans*; 7, *Enterococcus casseliflavus*; 8, *Enterococcus italicus*; 9, *Micrococcus luteus*; 10, *Staphylococcus haemolyticus*; and 11, *Aerococcus viridans*.

Online Resource Fig. 1

Online Resource Fig. 2. Repeatability of the REP-PCR typing assay with primer BoxA2R after analysing of three randomly-selected isolates (A, B, and C) in three independent experiments (1, 2, and 3). Below, dendrogram of similarity of the different typing patterns clustered by the UPGMA method using the Simple Matching coefficient. M, GeneRuler™.

Online Resource Fig. 2

Supplementary Figure 3

Supplementary Figure 3.- Typing REP-PCR profiles obtained with primer BoxA2R among the 36 *Enterococcus faecium* isolates from Koozeh cheese. Below, dendrogram of similarity of the different typing patterns clustered by the UPGMA method using the Simple Matching coefficient. M, GeneRuler™. The broken line denotes the arbitrary percentage of similarity used to consider isolates as different strains.

Online Resource Fig. 3