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ORIGINAL PAPER

Microbial diversity of the traditional Iranian cheeses Lighvan and Koozeh, as revealed by polyphasic culturing and culture-independent approaches

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Abstract The microbiota of two traditional Iranian cheeses (Lighvan and Koozeh) made of raw ewe's milk or mixtures of ewe's and goat's milk without starter addition was explored by culture-independent and culture-dependent approaches. Three batches of Lighvan and one of Koozeh were subjected to culture-independent polymerase chain reaction (PCR)–denaturing gradient gel electrophoresis (DGGE) analysis and sequencing of dominant bands to assess the microbial structure and dynamics through manufacturing and ripening. In addition, culturing in elective media for lactic acid bacteria (M17, MRS and KAA), isolation of single colonies (n=130), molecular identification by PCR-amplified ribosomal DNA restriction analysis and sequencing, and differentiation at the strain level by repetitive extragenic palindromic PCR was also performed. DGGE analysis showed that the dominant amplicons in all four cheese batches belonged to *Lactococcus lactis* and *Streptococcus parauberis*. In addition, *Escherichia coli* and *Lactococcus garvieae* were frequently identified in both Lighvan and Koozeh, while *Streptococcus*

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thermophilus was found occasionally. In contrast, *Enterococcus faecium* and *Enterococcus faecalis* were found to be dominant among the isolates in all batches. These species showed a high genetic diversity. The discrepancy between culturing and DGGE results suggested that dominant populations were in a nonrecoverable state in the used media. This reinforces the idea that culture-dependent and culture-independent techniques provide complementary data, ultimately affording a better description of cheese ecosystems. These data could be of help in the selection of commercial starters for industrial-scale manufacture of Lighvan and Koozeh cheeses using pasteurised milk. Alternatively, microbial analysis would allow the selection of appropriate strains for designing of specific starters for traditional cheese manufacture.

伊朗传统Lighvan和Koozeh干酪的微生物多样性

摘要采用纯培养和非培养方法研究了由生鲜羊奶或者混合羊奶(羊奶和山羊奶)自然发酵制作的伊朗传统Lighvan和Koozeh干酪的微生物区系。对3批Lighvan干酪和1批Koozeh干酪进行了非培养的PCR-DGGE分析和主要条带的测序, 以此评价干酪制作和成熟过程中微生物区系的结构和动力学。此外, 采用选择性培养基(M17, MRS and KAA)对乳酸菌进行了培养, 采用PCR-ARDRA、基因测序以及rep-PCR方法对分离出的单个菌落(n=130)从分子水平上进行鉴定。DGGE分析结果表明, 在所有4批干酪样品中优势菌群为*Lactococcus lactis* a和*Streptococcus parauberis*。此外, 在Lighvan和 Koozeh干酪中*Escherichia coli*和*Lactococcus garviea*的检出频率较高, 但只在几个干酪样品中检测到*Streptococcus thermophilus*。相反, 在所有样品中*Enterococcus faecium* 和*Enterococcus faecalis*也是主导菌群。微生物菌群之间表现出较高的生物多样性。纯培养和非培养的DGGE结果之间的差异表明主导菌群在这些培养基中是不可回收的, 这种结果说明在进行干酪微生物生态系统的研究中, 纯培养和非培养方法获得的数据可以互补。可以从获得的菌株中筛选出具有潜在工业化生产Lighvan和Koozeh干酪的发酵剂, 也可以从中筛选出特定的菌株作为发酵剂用于传统干酪的生产。

Keywords Traditional cheeses · Biodiversity, Lactic acid bacteria · Denaturing Gradient gel electrophoresis · DGGE

关键词 传统干酪 · 生物多样性 · 乳酸菌 · 变性梯度凝胶电泳 · DGGE

1 Introduction

Lighvan and Koozeh are among the best known and most appreciated of all traditional Iranian cheeses. They are manufactured in the neighbouring northwestern provinces of East and West Azerbaijan, respectively, from raw ewe's milk or a mixture of ewe's and goat's milk following ancient cheesemaking technologies without addition of starter. The main steps of their respective technologies are presented in Fig. 1. The cheeses demonstrate typicity in taste and flavour and their popularity is increasing at Iranian market.

Starter-free cheeses made from raw milk—such as Lighvan and Koozeh—rely for their acidification and ripening on the action of their indigenous lactic acid bacteria (LAB; Wouters et al. 2002). However, for such traditional cheeses to be competitive in national and international markets, product standardisation is necessary and food safety must be ensured. This requires the identification, characterisation and typing of the key microorganisms which grow in them, and the selection of those

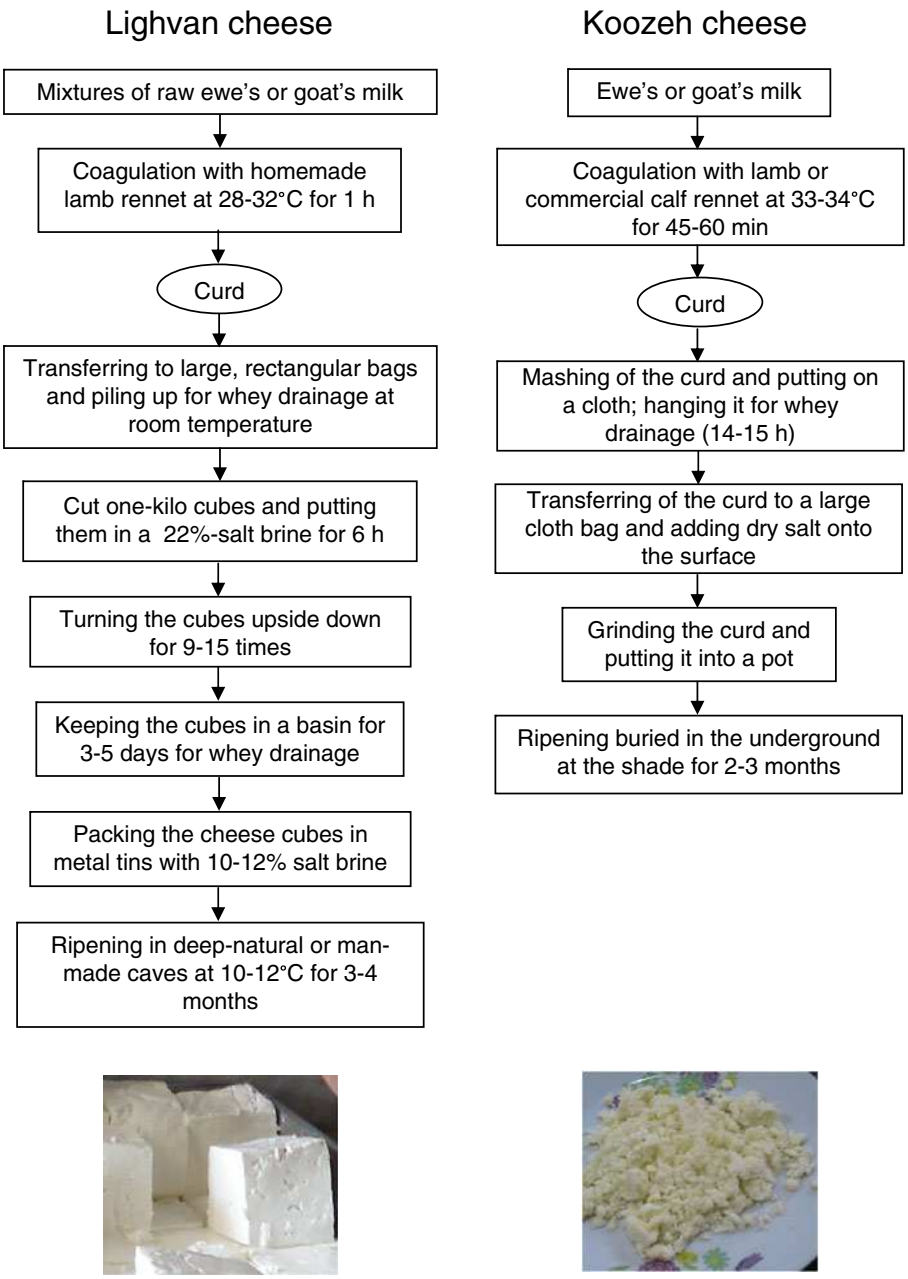

Fig. 1 Diagram and flow chart of manufacturing and ripening stages of traditional, Iranian raw milk cheeses Lighvan (**a**) and Koozeh (**b**)

appropriate for use as specific starters and adjunct cultures (Parente and Cogan 2004). The use of such starters would ensure that fermentations can be reliably reproduced while preserving the typical, traditional bouquet of these cheeses (Poznanski et al. 2004; Randazzo et al. 2008). The Lighvan and Koozeh ecosystems

may also harbour LAB strains with unique flavour-forming capabilities that might be advantageous in different areas of the dairy industry (Ayad et al. 2001) or in the production of new, broad-range, natural antimicrobials (Ayad et al. 2002).

The microbiota of fermented foods can now be monitored by a variety of molecular methods (Cocolin et al. 2007; Jany and Barbier 2008). Approaches combining classical culturing followed by molecular identification of the isolates and application of culture-independent molecular techniques are at present the most reliable choice to obtain objective results about microbial structure and dynamics of any ecosystem (Pogacic et al. 2010). The polymerase chain reaction (PCR) coupled with denaturing gradient gel electrophoresis (DGGE)—PCR–DGGE—is one such culture-independent molecular method that has been used to characterise the microbial populations of dairy products (Cocolin et al. 2002; Ercolini et al. 2001; Flórez and Mayo 2006; Lafarge et al. 2004). In fact, such characterisation has already been performed over the manufacture and ripening of a number of traditional cheeses (Coppola et al. 2001; Ercolini et al. 2004; Flórez and Mayo 2006; Pogacic et al. 2010; Randazzo et al. 2002).

Few microbial studies have been made on traditional Iranian cheeses. However, pioneering work has identified the dominant LAB species in ripened Lighvan cheese (Abdi et al. 2006; Barouei et al. 2008), as well as the cultivable lactobacilli that appear during manufacture and ripening (Kafili et al. 2009). To our knowledge, studies on the microbiota of Koozeh cheese have never been performed. In this study, PCR–DGGE was used to type the major microbial populations during the manufacture and ripening of three batches of Lighvan and one batch of Koozeh. In addition, a series of cultivable LAB strains were isolated and subjected to molecular identification and typing. The combined polyphasic approach allowed to assess the diversity and dynamics of the dominant microbiota associated with these two cheeses.

2 Materials and methods

The general experimental outline applied for the microbiological characterisation of Lighvan and Koozeh cheeses by culture-dependent and culture-independent approaches is depicted in Fig. 2.

2.1 Sampling

Samples of raw milk, curd and cheeses (three batches of Lighvan made by different producers and one batch of Koozeh) at different ripening periods (3, 7, 15, 30, 60 and 90 days) were sampled and transferred to the laboratory under refrigeration. Culturing analyses were performed within 6 h after arrival. Samples were then frozen at −20 °C until they were used to isolate total microbial DNA.

2.2 DGGE analysis of Lighvan and Koozeh cheeses

2.2.1 Extraction of total microbial DNA

Frozen samples were thawed at 4 °C and homogenised in 2% sodium citrate. Homogenates were then used for the isolation of total microbial DNA employing the

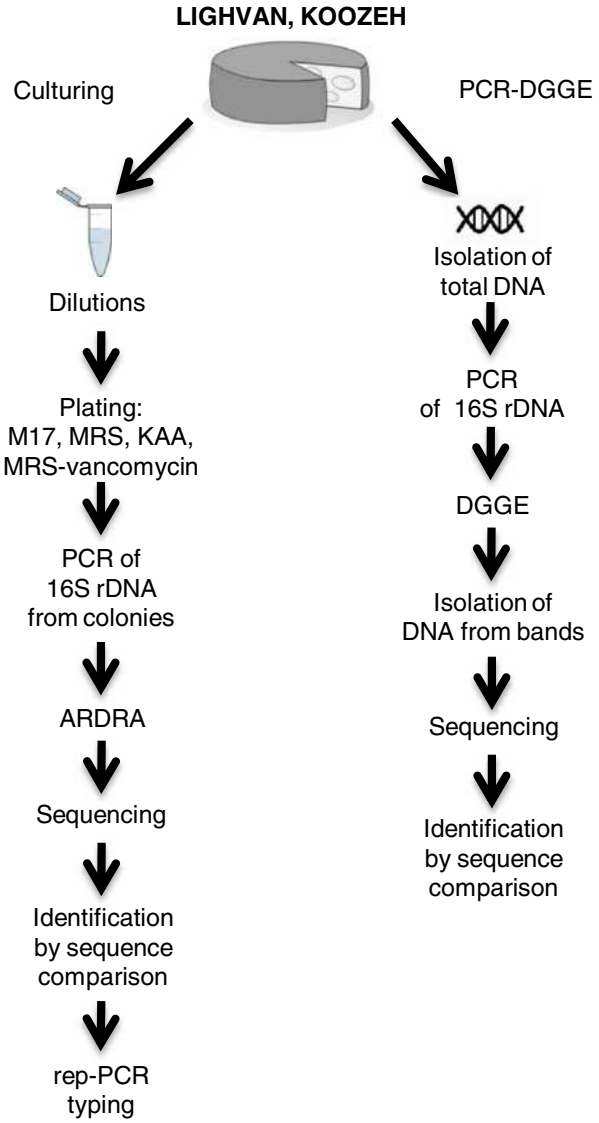

Fig. 2 Diagram of the experimental scheme applied for the microbiological characterisation of Lighvan and Koozeh cheeses

QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. DNA quantity and quality was measured by absorption at 260 and 280 nm using a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

2.2.2 PCR amplification of 16S rRNA sequences

One hundred nanogram of purified DNA was used as a template in PCR amplifications of the V3 region of the bacterial 16S rRNA gene using the universal primers F357-GC clamp (5′-TACGGGAGGCAGCAG-3′, to which a 39 bp GC sequence was linked) and R518 (5′-ATTACCGCGGCTGCTGG-3′), as reported by

Muyzer et al. (1993). Amplification of the D1 domain of the 26S rRNA gene of fungi was accomplished using primers NL1-GC (5′-GCCATATCAATAAGCGGAGGAAAG-3′, with a 39 bp GC clamp) and LS2 (5′-ATTCCCAAACAACTCGACTC-3′), as reported by Cocolin et al. (2002). PCR was performed in 50 μL reaction volumes containing 10 mmol.L^{-1} Tris-HCl, 50 mmol.L^{-1} KCl, 1.5 mmol.L^{-1} $MgCl_2$, 0.2 mmol.L^{-1} of each dNTP, 0.2 mmol.L^{-1} of the primers, and 5 U of Taq polymerase.

2.2.3 Electrophoretic conditions

DGGE was performed in a DCode apparatus (Bio-Rad) in 8% polyacylamide gels at 60 °C. After initial trials, the best separation of PCR amplicons was observed at denaturing gradients of 40–60% for bacteria and 30–50% for fungi. Electrophoresis was performed at 75 V for 16 h and 130 V for 4.5 h for bacterial and fungal amplifications, respectively. Bands were visualised under UV light after staining with ethidium bromide (0.5 μg.mL^{-1}) and photographed.

2.2.4 Identification of DGGE bands

Bands in the polyacrylamide gels were assigned to species by comparison with a control ladder of known strains (Flórez and Mayo 2006), namely *Lactococcus garvieae* CECT 4531^T, *Lactobacillus plantarum* CECT 748^T, *Leuconostoc mesenteroides* CECT 219^T, *Streptococcus parauberis* DSMZ 6631^T, *Enterococcus faecium* ATCC 19343^T, *Enterococcus faecalis* CECT 481^T, *Lactococcus lactis* subsp. *cremoris* MG 1363, *Escherichia coli* W3110, and *Lactobacillus paracasei* CECT 4022^T. To construct the ladder, purified DNA of the control strains was used in independent PCR–DGGE reactions and equal amounts of amplicons (50 ng.mL^{-1} each) were mixed before electrophoresis. DNA from bands that did not migrate to the positions of the controls was isolated by elution, re-amplified with the same primer pair without the GC clamp, sequenced by cycle extension in an ABI 373 DNA sequencer (Applied Biosystems, Foster City, CA, USA), and the sequences compared with sequences in the GenBank database using the Basic Local Alignment Search Tool programme (BLAST 2011) and with those held by the Ribosomal Database Project (RDP 2011). Sequences showing 97% similarity or higher were deemed to belong to the same species (Palys et al. 1997; Stackebrandt and Goebel 1994).

2.3 Culture-dependent approach

Milk samples were diluted in 0.1% sterile peptone water. Twenty five grams of curd and cheese samples at day 30 (fresh cheese) and at day 90 (ripened cheese) were homogenised in 225 ml of a sterile sodium citrate solution (2% *w*/*v*) using a Stomacher 400 (Seward, Worthing, UK). Dilutions of milk, curd and cheese samples were then plated in duplicate on agarified plates of M17 (Scharlab, Barcelona, Spain), MRS (Merck, Darmstad, Germany), and KAA (Oxoid, Basingstoke–Hampshire, UK) for enumeration and isolation of presumptive lactococci, lactobacilli and enterococci, respectively. Plates were incubated aerobically in a GasPack EZ system (BD, Franklin Lakes, NJ, USA) at either 30 °C (M17), 37 °C

(MRS) or 42 °C (KAA) for 24–72 h. Then, four to five representative colonies (according to shape, size and colour) from the highest dilutions were picked up at random, purified two or three times on the same media, and examined for Gram staining, catalase production and morphology. All Gram-positive, catalase-negative isolates were selected for further identification and stored frozen at −80 °C in MRS broth containing 20% glycerol.

2.4 Molecular identification of LAB species

Cryopreserved cultures were recovered in the corresponding media of isolation and identified by a polyphasic molecular procedure, including extraction of total DNA, partial amplification of 16S rRNA genes, amplified ribosomal DNA restriction analysis (ARDRA), sequencing and sequence comparison.

2.4.1 DNA extraction

For DNA isolation, single colonies were suspended in 50 μL of molecular grade water (Sigma-Aldrich, St. Louis, MO, USA), heated at 98 °C for 10 min in a thermocycler (Bio-Rad, Richmond, CA, USA), and centrifuged for 5 min at 16,000 rpm. Cell-free extracts were used as DNA templates for amplification of a major part of the 16S rRNA gene by PCR. Isolates that did not produce amplicon by this method were grown overnight in liquid media, centrifuged, washed in sterile saline (0.9%) and suspended in the same volume of water. Cell extracts were then obtained with glass beads (105 μm of diameter) in a Minibead Beater apparatus (Biospec Products, Bartlesville, OK, USA) and centrifuged as above.

2.4.2 Amplification of 16S rRNA genes

The primers used for the amplification of the 16S rRNA genes were 27FYM (5′-AGAGTTTGATYMTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′), based on the conserved regions of the 16S rRNA gene. As reported above, PCR was performed in 50 μL reaction containing 2 μL of each the cell-free extracts.

2.4.3 Amplified ribosomal DNA restriction analysis

Amplicons were subjected to ARDRA by digestion with the restriction enzymes *Hae*III and *Hha*I (Invitrogen Ltd., Paisley, UK). DNA fragments were separated by electrophoresis in 1.5% agarose gels at 75 V for 90 min. Gels were then stained and visualised as above.

2.4.4 Sequencing and sequence comparison

Representative amplicons of the different ARDRA profiles were sequenced using the primer 27FYM. On average, 850 bp were obtained per sequence and the sequences were compared as above.

2.5 Typing of isolates

All isolates were grouped by repetitive extragenic palindromic PCR (rep-PCR) typing using primer BoxA2R (5′-ACGTGGTTTGAAGAGATTTTCG-3′) and employing the amplification conditions of Koeuth et al. (1995). PCR products were then electrophoresed and visualised as above. Pattern similarity was expressed via the simple matching (SM) coefficient, and patterns were clustered by the unweighted pair group method using arithmetic averages (UPGMA).

3 Results

3.1 Microbial dynamics of Lighvan and Koozeh cheeses by DGGE

Three batches of Lighvan cheese manufactured by independent producers, plus one batch of Koozeh cheese were analysed by PCR–DGGE using universal primers to track both bacterial and eukaryotic populations (Figs. 3 and 4, respectively; Online Resource Table 1). The number of bacterial DGGE bands obtained for the different samples ranged between four (milk for Lighvan batch 2; Fig. 3b, line 1) and 11 (60 day-old Lighvan batch 1; Fig. 3a, line 7); all were identified at the species level. This large number of bacterial bands contrasts with the small number of eukaryotic populations; only two bands were observed in a 60-day-old sample of Lighvan (data not shown), and between two and four bands in samples of Koozeh over the entire experimental period (Fig. 4b). Eukaryotic bands were mostly identified at the genus level. In total, 25 bands (20 bacterial and five eukaryotic) were readily identified.

Similarities and differences in the DGGE profiles were noted between the two cheeses and between the distinct batches of Lighvan. The predominant bands for all cheeses over manufacture and ripening corresponded to the species *S. parauberis* (band d) and *L. lactis* (band g). In Lighvan, a band of variable intensity

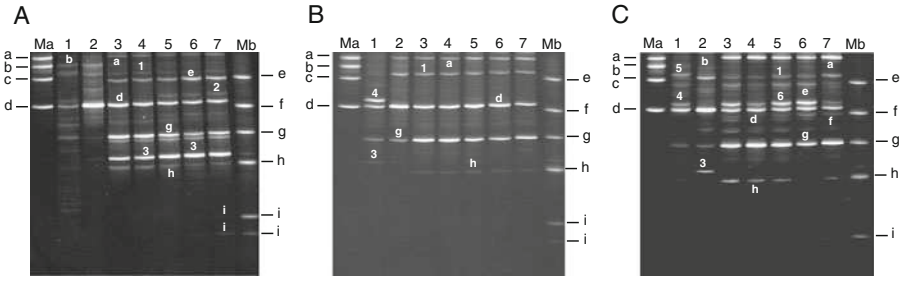

Fig. 3 Bacterial dynamics as shown by the DGGE profiles of the V3 variable region of the bacterial 16S rRNA gene in three independent batches of Lighvan cheese (**a**, **b**, and **c**, respectively) throughout manufacturing and ripening. Samples: milk, curd, and cheeses at days 3, 7, 15, 30, and 60 after manufacture. Ma and Mb, DGGE markers used as a control and composed of amplicons of isolated strains, as follows: *a L. garvieae*, *b L. plantarum*, *c L. mesenteroides*, *d S. parauberis*, *e E. faecium*, *f E. faecalis*, *g L. lactis*, *h E. coli*, and *i L. 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 L. raffinolactis*, *2 Lactococcus plantarum*, *3 S. thermophilus* (two bands), *4 S. haemolyticus*, *5 L. salivarius*, *6 M. caseolyticus*

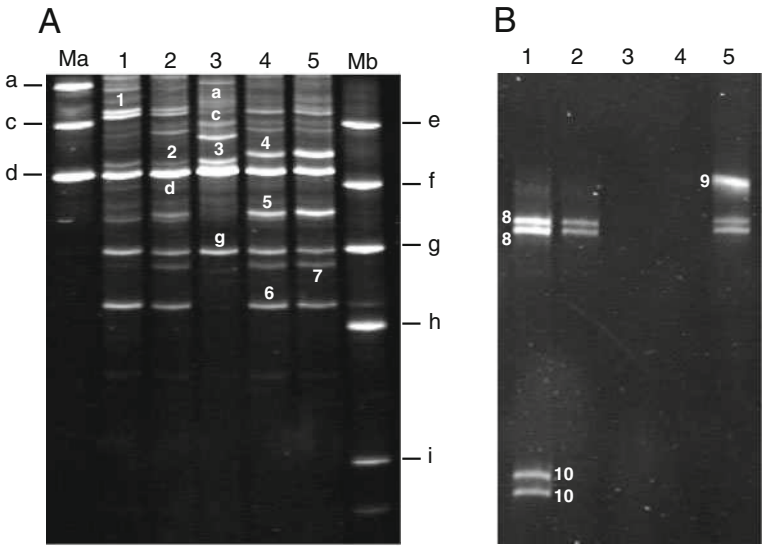

Fig. 4 Microbial dynamics in Koozeh cheese during manufacturing and ripening as judged from DGGE profiles of the V3 variable region of the bacterial 16S rRNA gene (**a**) and the D1 domain of the 26S rRNA gene of fungi (**b**) samples: cheese at 3, 7, 15, 30 and 60 days after manufacture. Ma and Mb as in Fig. 1 (except for absence of the *L. 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 L. raffinolactis*, *2 S. uberis*, *3 C. maltomaricum*, *4 L. curvatus*, *5 C. diazotrophica*, *6 S. thermophilus*; *7 V. tapetis*, *8 Warcupia* spp., *9 D. hansenii* (two bands) and *10 Penicillium* spp. (two bands)

corresponding to *L. garvieae* (band a) was observed in most cheese samples, as was a band for *E. coli* (band h). *Lactococcus raffinolactis* (band 1) and *E. faecium* (band e) were also present in two batches of Lighvan at most times. A band with a sequence matching that of *Streptococcus thermophilus* (band 3) was observed in batch 1 of Lighvan cheese at all sampling times and in the milk and curd samples respectively of the other two batches (Fig. 3). Bands identified occasionally included *L. plantarum* and *L. 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. 4). The DGGE profiles of the latter 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 days 3, 7, and 15 samples, *Carnobacterium maltomaricum* (band 3) was identified in day 15, *Lactobacillus curvatus* (band 4) in days 30 and 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. 4) and two bands each were identified as belonging to *Debaryomyces hansenii* (band 9) and *Penicillium* spp. (band 10; Fig. 4).

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

Counts (in colony forming units; cfu) on the different media and sampling points ranged widely. In general, counts in M17 and MRS were shown to be very similar and usually one logarithmic unit higher than those in KAA. The highest counts were recorded in curd samples (7.58 ± 0.15 cfu.g^{-1} in M17, 7.91 ± 0.01 cfu.g^{-1} in MRS and 6.44 ± 0.04 cfu.g^{-1} in KAA), decreasing one or two logarithmic units from that point onwards. As none of these three media is selective, isolated colonies were purified and subjected to a polyphasic molecular identification scheme. In total, 130 isolates (82 from Lighvan and 48 from Koozeh) were identified from the counting plates of M17 (47 isolates), MRS (59 isolates) and KKA (24 isolates) (Table 1). Regardless of

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				Medium of isolation (number of isolates)	Total
	Milk	Curd	30 days	90 days		
Lighvan cheese						
L. lactis subsp. lactis	3	1	–	–	M17 (4)	4
L. plantarum	3	8	9	–	MRS (20)	20
L. brevis	2	–	–	3	MRS (5)	5
E. faecium	3	11	11	13	M17 (18), MRS (15) KAA (5)	38
E. faecalis	–	2	–	9	KAA (7), M17 (4)	11
Enterococcus durans	1	–	–	–	MRS (1)	1
Enterococcus casseliflavus	–	–	1	–	M17 (1)	1
Entererococcus italicus	1	–	–	–	M17 (1)	1
Micrococcus luteus	–	–	–	1	MRS (1)	1
Total Lighvan	13	22	21	26	M17 (28), MRS (42) KAA (12)	82
Koozeh cheese						
E. faecium			7	29	M17 (13), MRS (13), KAA (10)	36
E. faecalis			–	5	M17 (4), MRS (1)	5
Enterococcus durans			–	1	M17 (1)	1
Enterococcus casseliflavus			–	2	KAA (2)	2
L. plantarum			1	–	MRS (1)	1
L. brevis			1	–	MRS (1)	1
S. haemolyticus			–	1	MRS (1)	1
Aerococcus viridans			–	1	M17 (1)	1
Total Koozeh			9	39	M17 (19), MRS (17) KAA (12)	48
Total	13	22	30	65	M17 (47), MRS (59) KAA (24)	130

the enzyme used (*Hae*III or *Hha*I), the isolates were grouped into 11 different ARDRA patterns (Online Resource Fig. 1). ARDRA profiles were compared with in silico analysis of several LAB species and with the profiles of the type strains used to construct the DGGE ladder. In addition, to unequivocally identify patterns at the species level, representative amplicons were sequenced and the sequences compared with those in the GenBank and RDP databases. Table 1 shows the molecular identification results. Nine different bacterial species were identified in Lighvan and eight in Koozeh. *Enterococcus* spp., including *E. faecium*, *E. faecalis*, *E. durans* and other species, made up the major part of the cultures. Of these, *E. faecium* (74 isolates) was dominant in both Lighvan and Koozeh at all sampling points, followed by *E. faecalis* (16 isolates). Enterococci species were isolated from all counting plates, indicating they constitute the dominant cultivable population (Table 1). Although in small numbers, *L. plantarum* (21 isolates) and *Lactobacillus brevis* (six isolates) were also recovered from both cheeses. In contrast to enterococci, lactobacilli were only identified from MRS agar plates (Table 1), suggesting they are subdominant populations in both Lighvan and Koozeh. In fact, counts recorded in MRS were usually slightly lower than those in M17. All isolates from the KKA medium were identified as *Enterococcus* spp. These results differed with those obtained in DGGE analysis since the species producing the most intense bands were almost absent in the cultures (only four *L. lactis* isolates from Lighvan cheese milk and curd were obtained, while *S. parauberis* isolates were never recovered).

Enterococcus spp. and *L. plantarum* isolates were all subjected to rep-PCR typing to evaluate intra-species diversity. Figure 5 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 SM coefficient. Given the reproducibility of the assay (around 90%; Online Resource Fig. 2), isolates sharing a percentage of similarity of >88% (an arbitrary figure) were considered to be the same strain (Fig. 5). 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). A wide genetic diversity was further 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 (Abdi et al. 2006; Kafili et al. 2009), and to our knowledge this is the first microbiological description of Koozeh cheese. A similar number of species was detected by molecular identification of the isolates and by the PCR–DGGE approach. Eight and nine different species were identified among the cultured isolates from Koozeh and Lighvan respectively, and four through 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 identified; they therefore provided complementary results (El-Baradei et al. 2007; Flórez and Mayo 2006; Poznanski et al. 2004; Randazzo et al. 2002) allowing a better description of these cheese ecosystems.

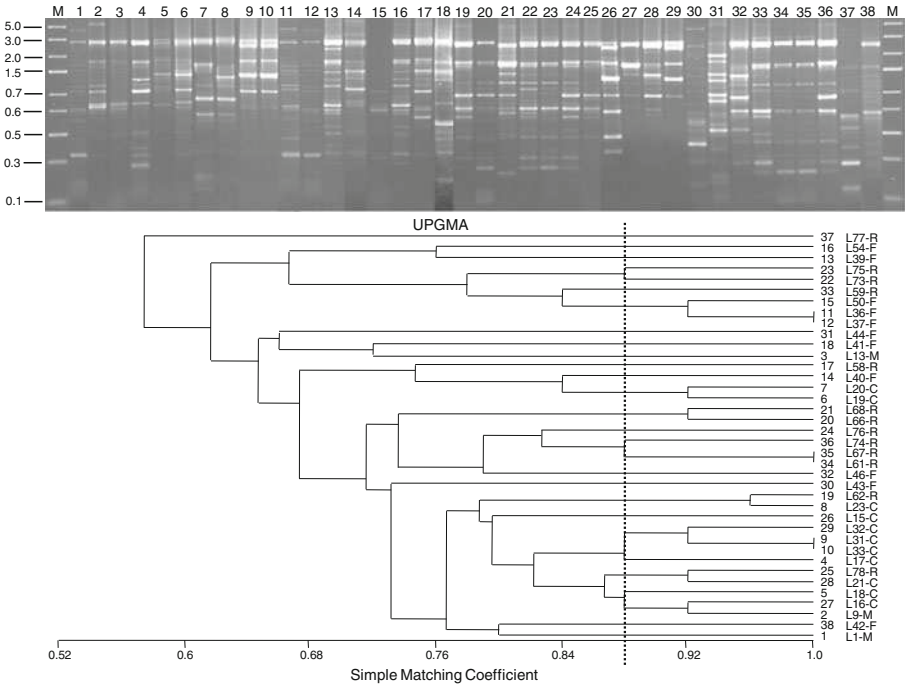

Fig. 5 Typing REP-PCR profiles obtained with primer BoxA2R among the 38 *E. faecium* isolates from Lighvan cheese. Below, dendogram of similarity of the different typing patterns clustered by the UPGMA method using the Simple Matching coefficient. *M* molecular weight marker GeneRuler^{TM} (Fermentas, 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)

The bacterial and fungal population dynamics recorded by DGGE for the two cheeses were similar to those reported for other traditional cheeses (Flórez and Mayo 2006; Pogacic et al. 2010; Randazzo et al. 2006). The bacterial diversity of dominant populations was usually greater in milk and curd samples, as some species were never identified in cheese (Table 1). Two to four high intensity bands were observed for the different batches of the two cheeses at all sampling times, which were accompanied by up to nine bands of lower intensity. The intensity of an individual DGGE band is assumed to be a semiquantitative measure of the corresponding microbe's abundance in the sample (Muyzer et al. 1993). In Lighvan and Koozeh, DGGE identified the dominant populations as belonging to *S. parauberis* and *L. lactis* species, with contributions at certain sampling times from *S. thermophilus* in both cheeses and *L. curvatus* in Koozeh. *Celerinatantimonas diazotrophica*, *L. garvieae*, *L. raffinolactis* and *E. coli* were found in most batches of the cheeses in subdominant numbers. Although *Enterococcus* spp. bands were found in several batches, high intensity bands of enterococcal species were only encountered in Lighvan cheese batch 1 (Fig. 3a). Thus, it was surprising to discover that species of this genus accounted for a large proportion of the cultured microorganisms (73.8% of the isolates; Table 1). These results, however, are not unusual, as DGGE bands corresponding to enterococcal species have been reported in some cheese types

(Coppola et al. 2001; Ercolini et al. 2003; Ogier et al. 2004) and not in others (Ercolini et al. 2004; Ogier et al. 2004; Randazzo et al. 2002). As enterococci were identified among isolates from the highest cultivable dilutions in all media, we must assume that the dominant species detected by PCR–DGGE are in a nonrecoverable state in the counting media utilised (although preferential lysis and/or DNA amplification cannot be excluded (Cocolin et al. 2007)). Thought nonselective, M17, MRS and KAA may be a highly stressful media, impeding growth of a part of the population, as has been reported elsewhere for other cheese systems when comparing count in M17 and MRS as compared to count on PCA (Alegría et al. 2009; Flórez and Mayo 2006). The paradigm in this study is KAA, which consistently showed counts one or two logarithmic units lower than those in the other two media, while enterococci strains were equally isolated from all three.

Of note in the sampled cheeses is the presence of bands related to *S. thermophilus* in most batches; these Iranian cheeses might therefore be considered a good source of new strains of this important cheese starter. DGGE bands of *S. thermophilus* have also recently been reported in a traditional Spanish starter-free cheese made from raw cow's milk (Alegría et al. 2009). The identified strains of *S. thermophilus* in the latter cheese have been isolated and are currently being characterised and compared with industrial starter strains (unpublished). Similarly, *L. garvieae*, a lactic acid bacterium similar to *L. lactis* (Fernández et al. 2010), has been identified by culturing and molecular methods in other cheese types (Alegría et al. 2009; Flórez and Mayo 2006).

The fact that cultivable strains belonging to the populations of the most prominent bands (*S. parauberis* and *L. lactis*) were not readily recovered on the enumeration plates strongly suggests that these microbial species are in a noncultivable state in Lighvan and Koozeh. Similar results have been reported elsewhere for other traditional cheeses (Ercolini et al. 2004; Randazzo et al. 2002). However, the DGGE results indicate that these two populations reach high densities at the beginning of the manufacturing process; certainly, they are present in the milk. *S. parauberis* has been associated with subclinical and clinical mastitis (Pitkälä et al. 2008); therefore, its presence in cheese is not desirable. In contrast, *L. lactis* is the typical LAB species of cheese. It enjoys 'generally regarded as safe' status and its enzymes contribute towards the typical taste and aroma profiles of cheese (Mayo 2010; Parente and Cogan 2004). Specific starters for these Iranian cheeses should therefore include strains of this species. Isolation of such *L. lactis* strains would have to be undertaken at the beginning of cheese manufacture (curdling of the milk, acidification, whey drainage, etc.).

Enterococci have been repeatedly reported to constitute major populations in artisanal, traditional cheeses made from raw milk (for a review, see Giraffa 2003). The high enterococci counts in Lighvan and Koozeh agree well with the low pH of these cheeses (average, 4.64) and the high concentration of salt present during ripening (up to 5.27% at day 90). Under such harsh conditions, *Enterococcus* spp. may thrive better than other LAB species. Though the presence of high numbers of enterococci in foods is controversial (Ogier and Serror 2008), strains of some species have been proposed as starters or adjunct cultures for several cheese types (Giraffa 2003).

A high intraspecies diversity was found among the enterococci isolates using rep-PCR, which suggests a high subsequent phenotypic diversity (Fig. 5). Moreover, several strains were shown to produce bacteriocins, such as enterocin A, B, P and X,

or a combination of these (unpublished). Certainly, enterococci strains play pivotal roles in the production of aroma compounds (El-Baradei et al. 2007; Foulquié Moreno et al. 2006; Giraffa 2003). However, for their safe use in foods, candidate strains would have to be subjected to a complete characterisation, guaranteeing the absence of recognised virulence factors and atypical, potentially transferable antibiotic resistances (Foulquié Moreno et al. 2006; Ogier and Serror 2008).

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

5 Conclusions

This work contributes to the microbiological characterisation of the most important traditional Iranian cheeses, Lighvan and Koozeh. The fact that some microbial populations were detected by one identification method only stresses the importance of combined approaches for fully describing the microbiota of naturally fermented cheeses. Microbial data could either help in the selection of appropriate commercial starters for industrial scale manufacture or in designing specific starters for traditional cheese manufacture. The results may further provide the basis for the future award of protected designation of origin status or an equivalent quality label for these cheeses.

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