International Journal of Dairy Technology SDT SOCIETY OF DAIRY TECHNOLOGY

doi: 10.1111/j.1471-0307.2011.00738.x

ORIGINAL RESEARCH

# The biodiversity and evolution of lactic flora during ripening of the Iranian semisoft *Lighvan* cheese

MOHAMMAD R EDALATIAN,[1] MOHAMMAD B HABIBI NAJAFI,[1]* ALI MORTAZAVI[1] and BALTASAR MAYO[2]

[1]*Department of Food Science & Technology, Faculty of Agriculture, Ferdowsi University of Mashhad, P. O. Box 91775-1163, Mashhad, Iran, and* [2]*Instituto de Productos Lácteos de Asturias (CSIC), Departamento de Microbiología y Bioquímica, 33300 Villaviciosa, Asturias, Spain*

*Ninety-five isolated strains of Lactic acid bacteria (LAB) were identified from* Lighvan *cheese. The LAB evolution showed the dominance of lactococci and lactobacilli in the first stage and substitution of these genera by enterococci at the end of ripening. The most predominant strains were* Enterococcus faecium *(22.44%),* Lactococcus lactis *ssp.* lactis *(20.4%),* Lactobacillus plantarum *(18.36%) and* E. faecalis *(14.28%), respectively. Eleven and 51 different carbohydrate fermentation profiles were observed according to API 20 STREP and API 50 CH, respectively. API 20 STREP dendogram showed identical fermentation profiles of some* E. faecalis *and* E. faecium *strains, indicating that these strains might be well adapted to the whole cheese manufacture.*

**Keywords** Lactic acid bacteria, *Lighvan*, Production stage, Raw milk cheese.

## INTRODUCTION

The impact of lactic flora on the sensory and physical properties of different raw milk cheeses has been investigated by others worldwide. The findings of such investigations aimed at industrialising traditionally produced raw milk cheeses. Investigation into the microbiota of Iranian raw milk cheeses has been addressed rarely (Barouei *et al.* 2008; Navidghasemizad *et al.* 2009). Lactic acid bacteria (LAB) play an important role in developing cheese flavour, whether added as a starter culture or present as an indigenous flora. That is why these bacteria are important from a technological point of view. Moreover, some particular LAB, such as *Enterococcus faecalis*, *E. faecium*, *Lactobacillus curvatus*, *L. paracasei* ssp. *paracasei* and *Lac. lactis* ssp. *lactis*, have been claimed to have the ability of bacteriocin production (Caridi 2003).

Owing to the consistent presence of enterococci genus in different types of cheeses, the impact and influence of enterococci on cheese manufacture has been investigated. There are some contradictory opinions about this group of LAB. Some researchers proved the positive effect of *E. faecalis* var. *liquefaciens* in the quality of Roquefort cheese (Devoyod 1969; Devoyod and Muller1969). Devoyod and Desmazeaud (1971) found a beneficial effect of enterococci on other LAB growth. On the other hand, bacteriocin production (enterocins) by enterococci has a controlling effect on pathogens and biogenic amines–producing bacteria such as some lactobacilli in cheese (Farias *et al.* 1994; Giraffa 1995; Aymerich *et al.* 1996; Joosten and Nunez 1996). In contrast, some negative effects of enterococci on cheese quality and health have been reported. Salvadori (1969) found the development of bitter flavour because of the application of *E. faecalis* as a starter in the production of Gorgonzola cheese. Because of their role in ripening, flavour development and bacteriocin production in cheese, it has been suggested that enterococci with desirable technological and metabolic traits could be included in starter cultures of various cheeses (Foulquie Moreno *et al.* 2006).

Technologically, the utilisation of starter cultures in cheese production will end up with consistent quality but it is most likely to result in product with limited flavour. On the other hand, consumers prefer dairy products with original taste. Hence, exploration of wild strains existing in nature and traditionally fermented foods is being of interest for manufacturing novel dairy products with original taste. Raw milk cheeses produced traditionally have this potential for the isolation of new strains for exploiting in dairy industry (Navidghasemizad *et al.* 2009).

*Author for correspondence. E-mail: habibi@um.ac.ir

**Table 1** Average results of basic chemical parameters of the cheese milk, curd and *Lighvan* cheese samples throughout manufacturing and ripening

| | *Stage during manufacturing and ripening* | | | | | |
|---|---|---|---|---|---|---|
| *Parameters* | *Milk* | *Curd* | *30 days* | *45 days* | *75 days* | *90 days* |
| Total solids (%) | 19.70 ± 2.98 | 25.23 ± 1.03 | 37.63 ± 1.10 | 37.86 ± 1.50 | 38.48 ± 0.91 | 40.10 ± 2.40 |
| Fat (%) | 7.94 ± 0.70 | 12.50 ± 0.91 | 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 | 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 | 5.30 ± 0.22 | 5.45 ± 0.28 | 5.33 ± 0.16 | 5.02 ± 0.32 |
| NaCl (%) | – | – | 3.84 ± 0.26 | 3.87 ± 0.38 | 3.96 ± 0.19 | 4.21 ± 0.43 |
| Average S/M (%) | – | – | 10.20 | 10.22 | 10.29 | 10.50 |

–, not determined; S/M, salt in moisture.

Among several raw milk cheeses in Iran, *Lighvan* cheese represents the most popular traditional cheese made from raw sheep's milk. The chemical composition of the cheesemilk being used in *Lighvan* cheese production is illustrated in Table 1 (Kafili *et al.* 2009). Somatic cells in such milk were <500 000/mL (data not shown). This cheese is categorised as a semisoft cheese with a desired sour taste, cream colour, high fat content and crumbly texture.

This study was carried out taking into account previously published works. In this study, the biodiversity of LAB and their evolution during four different stages mainly affecting cheese production were monitored, and more specific and accurate phenotypic methods, namely API 50CHL and API 20 Strep, were used to identify and classify LAB by constructing dendograms. To our knowledge, such studies on *Lighvan* cheese have not yet been attempted so far. However, this article does not provide any information on spoilage or pathogenic micro-organisms of *Lighvan* cheese: such information will be published in a further paper.

The objective of this study was to explore and identify the native LAB and their evolution in *Lighvan* cheese using traditional and culture-dependent methods, especially API 50 CHL and API 20 STREP Kits with the aim of introducing a starter cultures with known, predictable and stable characteristics for producing *Lighvan* cheese with typical flavour on an industrial scale, because future growth and economic vitality of dairy industry depend on starter cultures.

## MATERIALS AND METHODS

### Sampling

Samples were collected from the region (*Lighvan* valley, Tabriz, East Azerbaijan, Iran) from 10 local dairy plants randomly in summer (2009). Samples were taken from four different stages (milk, curd, fresh cheese 1 day old and ripened cheese 3 months old). Samples were then transferred to the laboratory under refrigeration.

### Cheese manufacture

*Lighvan* cheese is produced in a village with the same name in a region of East Azerbaijan province in some small local dairies (Kafili *et al.* 2009; Navidghasemizad *et al.* 2009). Milk was cooled to 28–32 °C depending on the season, immediately after milking. No starter was used in the production of this cheese, and only natural lamb rennet or commercial microbial rennet was added for the curdling of milk. The curd was then cut and drained using cheesecloth and pressure. The produced curd was put in high salt brine (22%) for 24 h, followed by a ripening in less salted brine (12%) at 10–12 °C for at least 3 months. Ripening period took place in underground caves that naturally have cold temperature.

### Isolation and identification of isolates

*Preparation of milk, curd and cheese samples*

For milk samples, decimal dilutions were prepared directly in 0.1% sterile peptone water, and regarding the curd and cheese samples, 25 g sample was added to 225 mL sterile sodium citrate solution (2% w/v). This mixture was transferred to a stomacher bag and was homogenised in a Stomacher homogeniser (Type 400; Seward, London, UK). Isolation and colony counts were made on the following media: MRS Agar (Merck) for the isolation of lactobacilli, M17 for lactococci, MRS Agar + vancomycin (20 μg/mL) for leuconostocs and Kanamycin aesculin azid agar (KAA) for enterococci. The aforementioned process for all strains was carried out in duplicate. Finally, plates were incubated anaerobically (Gas-pack system) at 30, 37 and 42 °C. The incubation period varied between 24–48 and 72 h depending on the bacteria groups. Counting was conducted only for those plates containing 30–300 colonies and from plates corresponding to the highest dilution. Four to five different colonies (according to shape, size and colour) were selected randomly. Then, they were purified two or three times on the same media. Single colonies from each plate were examined by Gram staining and for catalase production and microscopic morphology. Finally, only Gram-positive, catalase-negative isolates were considered and stored in MRS broth containing 20% glycerol and were freeze-dried. Totally, about 100 isolates from different samples (milk, curd, fresh and ripened cheese) were subjected to further biochemical and confirmatory tests.

*Biochemical and confirmatory tests*

After confirming the catalase-negative and Gram-positive tests for each single and pure colony, morphology of bacilli or cocci was determined by microscopic observation. Then, growth at 10 and 45 °C, growth at 6.5% salt, pH = 9.6, L-arginine hydrolysis with Nessler's reagent, aesculin hydrolysis, production of CO2 from glucose in MRS broth (with Durham tubes), citrate utilisation in Simon citrate Agar and Voges–Proskauer test in MR-VP medium were conducted as confirmatory tests.

Gram-positive, catalase-negative homofermentative cocci capable of growing at 10 °C and pH 9.6 but not at 45 °C or in 6.5% NaCl were considered as lactococci. For typing to species level, API 50 CHL strips with 50 CHL medium (Biomeriux, Montalieu-Verciue, France) were used. After identification of all isolates at genus level with biochemical and confirmatory tests, carbohydrate fermentation test was performed using API 50 CH for colonies from MRS, MRS + vancomycin and M17 and API 20 STREP for colonies from KAA according to manufacturer's procedure.

*Carbohydrate fermentation profile:* The fermentation profile of 49 sugars and polyalcohols was recorded using the API 50-CHL system (BioMerieux) according to the manufacturer's instructions. Also API 20 STREP system was used for those isolates that have been confirmed as enterococci by the confirmatory tests.

## Statistical analysis

Profiles obtained with a given technique were compared to each other by the simple matching coefficient and clustered using the unweighted pair groups average linkage analysis (UPGMA) (Multi-variate Statistical Package program).

## RESULTS AND DISCUSSION

The distribution of LAB isolated from different media for *Lighvan* (at four steps) is presented in Table 2. This table gives us some information about the selectivity and suitability of the different media for LAB. MRS agar has been suitable for lactobacilli, and this genus was significantly dominant (22/33). Similar findings were also reported for Tulum cheese (Gurses and Erdogan 2006). On the other hand, other genus such as *Lactococcus* (3/33), *Enterococcus* (4/33), *Pediococcus* (3/33) and *Leuconostoc* (1/33) were found in this medium. Other workers have also reported the growth of *Lactobacillus* and *Lactococcus* more than other genera in this medium, which confirms the usefulness of MRS for isolating species of this genus (Lopez-Diaz *et al.* 2000). More than half (22 of 33) of the isolates on MRS medium were lactobacilli, and the others were coccal-shaped cells. The growth of cocci in MRS can be explained by the low selectivity of this medium, as it has been reported elsewhere (Caridi 2003).

Fox *et al.* (2000) reported the good selectivity of M17 agar for the isolation of lactococci. In our study, the majority of strains isolated from M17 were also lactococci (18/30). In contrast to our results, Navidghasemizad *et al.* (2009) showed that the most of the strains isolated from M17 medium were enterococci. Almost all of the 30 isolates on M17 agar were coccal-shaped LAB, including *Lactococcus* (18/30), *Enterococcus* (10/30) and *Pediococcus* (1/30). The remaining isolate was *Lactobacillus* (1/30). The usefulness of this medium has been proven by other researchers (Lopez-Diaz *et al.* 2000).

Kanamycin aesculin azid agar showed high selectivity for *Enterococcus* spp. (18/22). The majority of enterococci were isolated from PCA agar from Tulum cheese (Gurses and Erdogan 2006).

MRS supplemented with vancomycin is a suitable and selective medium for *Leuconostoc* spp. because of the high resistance of *Leuconostocs* against this antibiotic. However, other LAB such as many lactobacilli species can grow on this medium (Table 2) (Fox *et al.* 2000).

As shown in Table 3, in MRS medium, the number of colonies increased from milk to curd and after that decreased until ripened cheese. In MRS + vancomycin, no consistent increasing or decreasing trend from milk to the ripened cheese was observed. In M17, there was an increasing trend from milk to curd only at 37 °C. In KAA, the increasing trend was seen from milk to fresh cheese and after that this trend decreased to ripened cheese at 45 °C.

**Table 2** Distribution of LAB isolated from different media during processing from milk to ripened *Lighvan* cheese

| | *Media and incubation temperature(°C)* | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *MRS* | | | *MRS + vancomycin* | | | *M17* | | | *KAA* | | | |
| *Genus* | *30* | *37* | *45* | *30* | *37* | *45* | *30* | *37* | *45* | *30* | *37* | *45* | *Total* |
| *Lactobacillus* | 10 | 10 | 2 | 6 | 3 | – | 1 | – | – | – | – | – | 32 |
| *Lactococcus* | 1 | 2 | – | – | – | – | 4 | 7 | 7 | 2 | 1 | 1 | 25 |
| *Enterococcus* | 2 | 2 | – | – | – | – | 4 | 2 | 4 | 5 | 4 | 9 | 32 |
| *Pediococcus* | 2 | 1 | – | – | – | – | – | 1 | – | – | – | – | 4 |
| *Leuconostoc* | – | 1 | – | 1 | – | – | – | – | – | – | – | – | 2 |
| Total | 15 | 16 | 2 | 7 | 3 | – | 9 | 10 | 11 | 7 | 5 | 10 | 95 |

LAB, lactic acid bacteria.

**Table 3** Log colony-forming unit (cfu/mL and Log cfu/g) and standard deviation of lactic acid bacteria present in *Lighvan* cheese at four different stages (milk, curd, fresh and ripened cheese)

| *Media* | *Incubation temperature (°C)* | *Product* | | | |
|---|---|---|---|---|---|
| | | *Milk* | *Curd* | *Fresh cheese* | *Ripened cheese* |
| MRS | 30 | 6.82 ± 0.02[a] | 7.68 ± 0.3 | 6.50 ± 0.04 | 5.92 ± 0.03 |
| | 37 | 7.08 ± 0.12 | 7.91 ± 0.01 | 7.86 ± 0.01 | 6.62 ± 0.03 |
| | 45 | 4.65 ± 0.04 | 5.82 ± 0.02 | 5.86 ± 0.24 | <1 |
| MRS + vancomycin | 30 | 6.10 ± 0.09 | 5.57 ± 0.04 | 7.12 ± 0.07 | 5.64 ± 0.06 |
| | 37 | 6.28 ± 0.15 | 5.74 ± 0.07 | 7.36 ± 0.06 | 6.07 ± 0.09 |
| | 45 | <1[a] | <1 | <1 | <1 |
| M17 | 30 | 6.43 ± 0.05 | 7.58 ± 0.15 | 6.43 ± 0.05 | 7.08 ± 0.12 |
| | 37 | 6.56 ± 0.07 | 7.2 ± 0.14 | 7.08 ± 0.12 | 7.38 ± 0.12 |
| | 45 | <1 | <1 | 6.88 ± 0.16 | 7.62 ± 0.03 |
| KAA | 30 | 6.55 ± 0.06 | 6.67 ± 0.02 | 6.24 ± 0.1 | <6 |
| | 37 | 5.88 ± 0.16 | 6.23 ± 0.09 | 5.79 ± 0.14 | <6 |
| | 45 | 6.00 ± 0.23 | 6.44 ± 0.04 | 6.52 ± 0.02 | 5.08 ± 0.12 |

KAA, Kanamycin aesculin azid agar.

[a]Log colony-forming unit (Log cfu/mL, Log cfu/g) in these cases was lower than 1.

Std (standard deviation of two replicates from each observation).

**Table 4** Biochemical characteristics of the LAB isolated from *Lighvan* cheese at different stages from milk to ripened cheese

| *Strains* | *Growth at 15 °C* | *Growth at 45 °C* | *Growth at 6.5% NaCl* | *Growth at pH 9.6* | *$CO_2$ from glucose* | *VP test* | *Arginine hydrolysis* | *Citrate utilisation* |
|---|---|---|---|---|---|---|---|---|
| *Lactobacillus plantarum* | + | − | +/− | + | − | − | − | − |
| *L. paracasei* ssp. *paracasei* | + | − | − | + | − | − | − | − |
| *L. brevis* | + | − | − | +/− | + | − | + | − |
| *L. delbrueckii* ssp. *delbrueckii* | − | + | + | + | − | − | − | − |
| *L. fructivorans* | − | +/− | − | + | − | − | − | − |
| *Lactococcus lactis* ssp. *lactis* | + | − | − | − | − | + | + | − |
| *Lac. lactis* ssp. *cremoris* | + | − | − | − | − | + | + | + |
| *Leu. lactis* | + | − | − | − | + | + | − | − |
| *Leuconostoc mesenteroides* | + | − | +/− | − | + | − | − | − |
| *Pediococcus pentosaceus* | + | − | + | − | − | + | + | − |
| *Enterococcus faecalis* | + | + | + | + | − | + | + | − |
| *E. faecium* | + | + | + | + | − | + | + | − |
| *E. durans* | + | + | + | + | − | + | + | − |

+, positive reaction; −, negative reaction; LAB, lactic acid bacteria.

Table 4 shows the biochemical characterisation of the LAB isolates from different stages of *Lighvan* cheese. Among the *lactobacilli*, only one species could grow at high temperature and 6.5% salt. All *lactobacilli* showed the ability of growth at pH 9.6, and none of them grew in the VP medium. The only heterofermentative *Lactobacillus* species was *L. brevis*. Among the cocci, only enterococci showed the ability of growth at high temperatures and at pH 9.6. All of the cocci showed growth at 15 °C. *Lactococcus lactis* ssp. *lactis* and *Leuconostoc lactis* did not grow at high concentration of salt (6.5%). Two heterofermentative species were *Leu. lactis* and *Leu. mesenteroides*. All of the cocci could grow in the VP medium except *Leu. mesenteroides*. Arginine hydrolysis was negative for *Leu. lactis* and *Leu. mesenteroides*.

Figure 1 shows the evolution of each genus during different stages in order to help us to understand the role of each of these species. In milk, enterococci were dominant and after that lactobacilli and lactococci followed by pediococci with lower proportions. During the ripening in next stages, enterococci and to a lesser extent lactobacilli showed the increasing trend but lactococci became lower. This phenomenon sounds logical because in most types of cheeses, similar trend has been seen. In first stage of processing, lactococci that are the responsible for milk acidification are predominant and progress until the

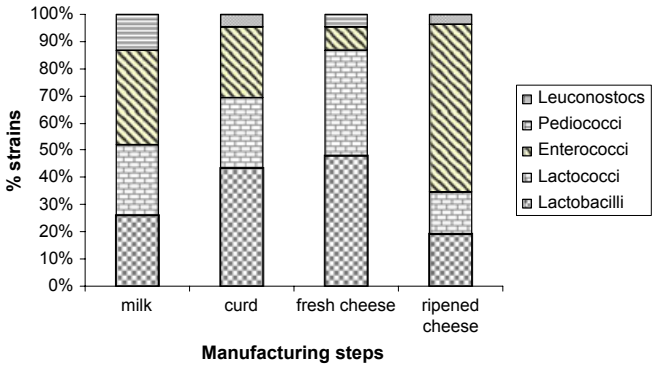


**Figure 1** Evolution of genera of lactic acid bacteria during manufacture of *Lighvan* cheese.

formation of curd. In following steps, the other LAB such as enterococci and lactobacilli become dominant.

Table 5 shows the distribution of LAB species found in our cheese samples in different stages of production. *Lactobacillus* genus (33.68%) comprised the *L. plantarum* (20.00%) the most dominant, followed by *L. paracasei* ssp. *paracasei* (7.36%), *L. brevis* (3.15%), *L. delbrueckii* ssp. *delbrueckii* (2.10%) and *L. fructivorans* (1.05%) in all production stages. Generally, *Lactobacillus* genus had an increasing trend from milk (26.08%) to curd (43.47%) and thereafter presented a declining trend until the ripening stage (19.23%). *Lactobacillus plantarum* was the only species identified in all stages. *Lactobacillus brevis* did not change significantly during the ripening stage. Gurses and Erdogan (2006) showed similar results for *L. brevis* and *L. curvatus* in their research. *Leuconostoc mesenteroides* and *Leu. lactis* were detected in curd and ripened cheese, respectively. *Lactococcus lactis* ssp. *lactis* constituted 26.08% of the micro-organisms in milk and curd samples, and after that they decreased in ripened cheese. Similar trend was seen in Tulum cheese (Gurses and Erdogan 2006). *Pediococcus pentosaceus* was found only in milk and fresh cheese stages. Finally, a wide variety of *Enterococcus* was found in our samples, particularly *E. faecalis* and *E. faecium* were the predominant species (15.78%). *Enterococcus faecalis* is a common species that isolated frequently from different cheese varieties such as Feta, Manchego, Teleme, Comte, Fontina, Serra and Cebreiro (Centeno *et al.* 1999; Lopez-Diaz *et al.* 2000; Sarantinopoulos *et al.* 2002; Giraffa 2003; Marino *et al.* 2003).

## Carbohydrate fermentation profiles

Table 6 shows some of the selected colonies that were examined phenotypically using API 20 STREP. As seen, aesculin hydrolysis and L-arginine hydrolysis for all enterococci were positive. Acid production from D-ribose, D-lactose and D-trehalose for all of the selected *E. faecalis*, *E. faecium* and *E. durans* was positive. None of the enterococci could produce acid from glycogen. For other carbohydrates, different species showed different fermentation profile. Only *E. faecalis* could produce acid from D-sorbitol (15/15) and starch (9/15) (Table 6).

**Table 5** Distribution of LAB during ripening from milk to ripened 3 months *Lighvan* cheese (according to phenotypic characterisation)

| | *Production stages* | | | | |
|---|---|---|---|---|---|
| *Type of lactic acid bacteria* | *Milk (%)* | *Curd (%)* | *Fresh cheese (1-day-old cheese) (%)* | *Ripened cheese (3-month-old cheese) (%)* | *Total number (%)* |
| *Lactobacillus* spp. | 6 (26.08) | 10 (43.47) | 11 (47.82) | 5 (19.23) | 32 (33.68) |
| *L. plantarum* | 4 (17.39) | 5 (21.73) | 8 (34.78) | 2 (7.69) | 19 (20.00) |
| *L. brevis* | 1 (4.34) | – | 1 (4.34) | 1 (3.84) | 3 (3.15) |
| *L. paracasei* ssp. *paracasei* | 1 (4.34) | 3 (13.04) | 1 (4.34) | 2 (7.69) | 7 (7.36) |
| *L. delbrueckii* ssp. *delbrueckii* | – | 2 (8.69) | – | – | 2 (2.10) |
| *L. fructivorans* | – | – | 1 (4.34) | – | 1 (1.05) |
| *Leuconostocs* spp. | – | 1 (4.34) | – | 1 (3.84) | 2 (2.10) |
| *Leuconostoc mesenteroides* | – | 1 (4.34) | – | – | 1 (1.05) |
| *Leu. lactis* | – | – | – | 1 (3.84) | 1 (1.05) |
| *Lactococcus* spp. | 6 (26.08) | 6 (26.08) | 9 (39.13) | 4 (15.38) | 25 (26.31) |
| *Lac. lactis* ssp. *lactis* | 6 (26.08) | 6 (26.08) | 8 (34.78) | 4 (15.38) | 24 (25.26) |
| *Lac. lactis* ssp. *cremoris* | – | – | 1 (4.34) | – | 1 (1.05) |
| *Pediococcus* spp. | 3 (13.04) | – | 1 (4.34) | – | 4 (4.21) |
| *Pediococcus pentosaceus* | 3 (13.04) | – | 1 (4.34) | – | 4 (4.21) |
| *Enterococcus* spp. | 8 (34.78) | 6 (26.08) | 2 (8.69) | 16 (61.53) | 32 (33.68) |
| *Enterococcus faecium* | 3 (13.04) | 2 (8.69) | 1 (4.34) | 9 (34.61) | 15 (15.78) |
| *E. faecalis* | 5 (21.73) | 4 (17.39) | 1 (4.34) | 5 (19.23) | 15 (15.78) |
| *E. durans* | – | – | – | 2 (7.69) | 2 (2.10) |
| Total | 23 (100) | 23 (100) | 23 (100) | 26 (100) | 95 (100) |

LAB, lactic acid bacteria.

**Table 6** Phenotypical profiles of some *Enterococci* and *Lactococci* isolated from different stages of *Lighvan* cheese according to API 20 STREP

| *Characteristics* | Enterococcus faecalis *(15)*[a] | E. faecium *(15)* | E. durans *(2)* | Lactococcus lactis *ssp.* cremoris *(1)* | L. lactis *ssp.* lactis *(3)* |
|---|---|---|---|---|---|
| Aesculin hydrolysis | 15[b] | 15 | 2 | 0 | 3 |
| L-Arginine hydrolysis | 15 | 15 | 2 | 0 | 3 |
| Growth on NaCl 6.5% | 15 | 15 | 2 | 0 | 0 |
| Growth at 45 °C | 15 | 15 | 2 | 0 | 0 |
| Acid from | | | | | |
| D-Ribose | 15 | 15 | 2 | 1 | 3 |
| L-Arabinose | 4 | 14 | 0 | 0 | 1 |
| D-Mannitol | 15 | 15 | 0 | 1 | 3 |
| D-Sorbitol | 15 | 4 | 0 | 0 | 0 |
| D-Lactose | 15 | 15 | 2 | 1 | 3 |
| D-Trehalose | 15 | 15 | 2 | 1 | 3 |
| Inulin | 0 | 4 | 0 | 0 | 0 |
| D-Raffinose | 0 | 6 | 0 | 0 | 0 |
| Starch | 9 | 0 | 0 | 0 | 0 |
| Glycogen | 0 | 0 | 0 | 0 | 0 |

[a]Number of strains investigated.
[b]Number of positive strains.

Among all of the colonies from different media, one *Lac. lactis* ssp. *cremoris* and three *Lac. lactis* ssp. *lactis* isolates were detected using API 20 STREP.

In total, 11 different carbohydrate fermentation profiles were observed (Fig. 2). When subjected to statistical analysis, they showed a high degree of similarity. The dendogram obtained

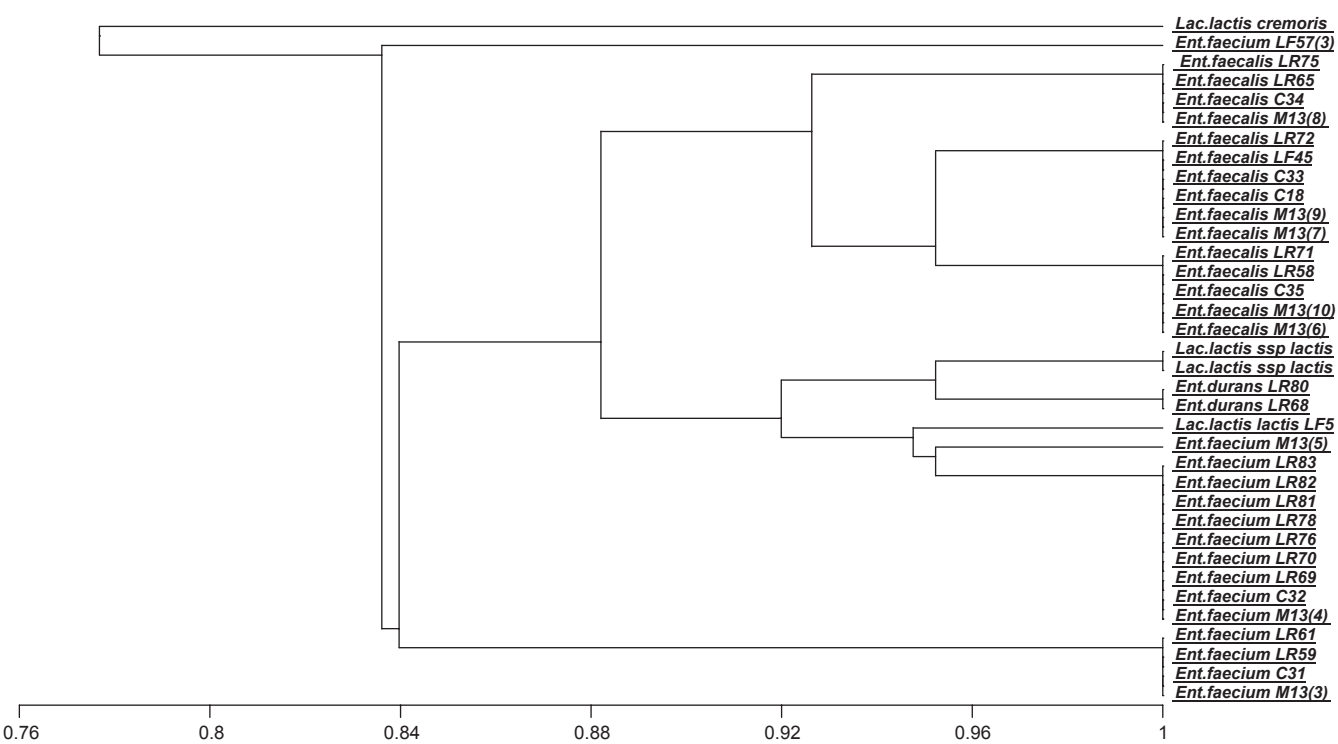


**Figure 2** Homology tree dendogram of the carbohydrate fermentation profiles obtained with the API 20 STREP system. The similarity matrix of the profiles was subjected to cluster analysis by the unweighted pair groups average linkage analysis clustering method (using the simple matching coefficient). Vertical lines of the dendogram represent the degree of similarity shared by the groups connected by the lines. Codes: M (Milk), C (Curd), LF (*Lighvan* Fresh cheese), LR (*Lighvan* Ripened cheese).

**Table 7** Carbohydrate fermentation differences between species obtained with the API 50-CHL system

| | *Number of isolates in all stages of production* | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| *Sugar profiles* | Lactobacillus plantarum *(18)* | L. brevis *(3)* | L. paracasei *ssp.* paracasei *(7)* | L. delbrueckii *ssp.* delbrueckii *(2)* | L. fructivorans *(1)* | Leuconostoc mesenteoides *(1)* | L. lactis *(1)* | L. lactis *ssp.* lactis *(20)* | Pediococcuc pentosaseus *(4)* |
| Glycerol | (1)[a] | (1) | (2) | − | − | − | − | (4) | (2) |
| D-Arabinose | − | − | 1 | (1) | − | − | − | − | − |
| L-Arabinose | (11) | + | (2) | − | − | − | − | (15) | (1) |
| D-Ribose | + | + | + | − | − | − | − | (19) | + |
| D-xylose | − | (2) | − | − | − | − | − | (1) | − |
| D-Galactose | + | + | + | + | − | − | + | + | + |
| D-Glucose | (17) | + | + | − | + | + | + | + | + |
| D-Fructose | + | + | + | − | − | − | + | + | + |
| D-Mannose | + | (2) | + | + | − | − | + | + | + |
| L-Sorbose | − | − | (1) | − | − | − | − | − | − |
| L-Rhamnose | (1) | − | − | − | − | − | − | (1) | − |
| Dulcitol | (1) | − | − | − | − | − | − | − | − |
| Inositol | − | − | (1) | − | − | − | − | − | − |
| D-Mannitol | (16) | (2) | + | − | − | − | − | (13) | (1) |
| D-Sorbitol | (7) | (1) | (4) | − | − | − | − | (3) | − |
| MDM[b] | (6) | − | − | − | − | − | − | (1) | − |
| MDG[c] | (2) | (1) | − | − | − | − | − | − | − |
| NAG[d] | (17) | + | + | − | − | + | + | + | + |
| Amygdalin | (14) | (2) | + | − | − | − | − | (13) | + |
| Arbutine | + | (2) | + | − | − | − | − | + | + |
| Esculine | + | + | + | − | − | + | − | + | + |
| Salicine | + | (2) | + | − | − | − | + | + | (3) |
| D-Cellobiose | (17) | (2) | + | + | − | − | − | + | + |
| D-Maltose | + | + | + | − | − | − | + | + | + |
| D-Lactose | + | + | (6) | − | − | + | + | + | + |
| D-Melibiose | (11) | (2) | (1) | − | + | − | + | (10) | (1) |
| D-Saccharose | (8) | (1) | (6) | − | − | − | + | (12) | (1) |
| D-Trehalose | (12) | (1) | (7) | − | − | − | − | (16) | + |
| Inulin | (1) | − | (1) | − | − | − | − | − | − |
| D-Melizitoze | (9) | (2) | (5) | − | − | − | − | (3) | − |
| D-Raffinose | (3) | − | − | − | − | − | − | − | − |
| Amidon | (1) | − | − | − | − | − | − | (2) | − |
| Glycogene | − | − | − | − | − | − | − | (2) | − |
| Xylitol | − | − | − | − | − | − | − | (2) | − |
| Gentiobiose | (16) | (2) | (7) | − | − | − | − | (15) | + |
| D-Turanose | (1) | − | (3) | − | − | − | − | − | − |
| D-Tagatose | (5) | (1) | (6) | − | − | − | − | (8) | + |
| L-Arabitol | − | − | (1) | − | − | − | − | − | − |
| Gluconate | (3) | (1) | (3) | − | − | − | − | (5) | − |
| 5-Ketogluconate | − | (1) | − | − | − | − | − | − | − |

+, positive reaction; −, negative reaction, none of the isolates fermented, erythritol, L-xylose, adonitol, methyl-βD-xylopyranoside (MDX), D-lyxose, D-fucose, L-fucose, D-arabitol, 2-ketogluconate.

[a]The numbers in parentheses correspond to the number of positive isolates in each test.

[b]Methyl-αD-mannopyranoside.

[c]Methyl-αD-glucopyranoside.

[d]*N*-acetyl glucosamine.

after UPGMA analysis is shown in Fig. 2. We can conclude that those isolates or strains have identical vertical line; they have similar carbohydrate fermentation profiles. Also, this dendogram shows that some strains from different production stages have been located in the same vertical line (e.g. *E. faecalis* LR72, *E. faecalis* LF45, *E. faecalis* C33 and *E. faecalis* M13). This means that these strains can survive during the manufacture, so we can apply them as starter cultures.

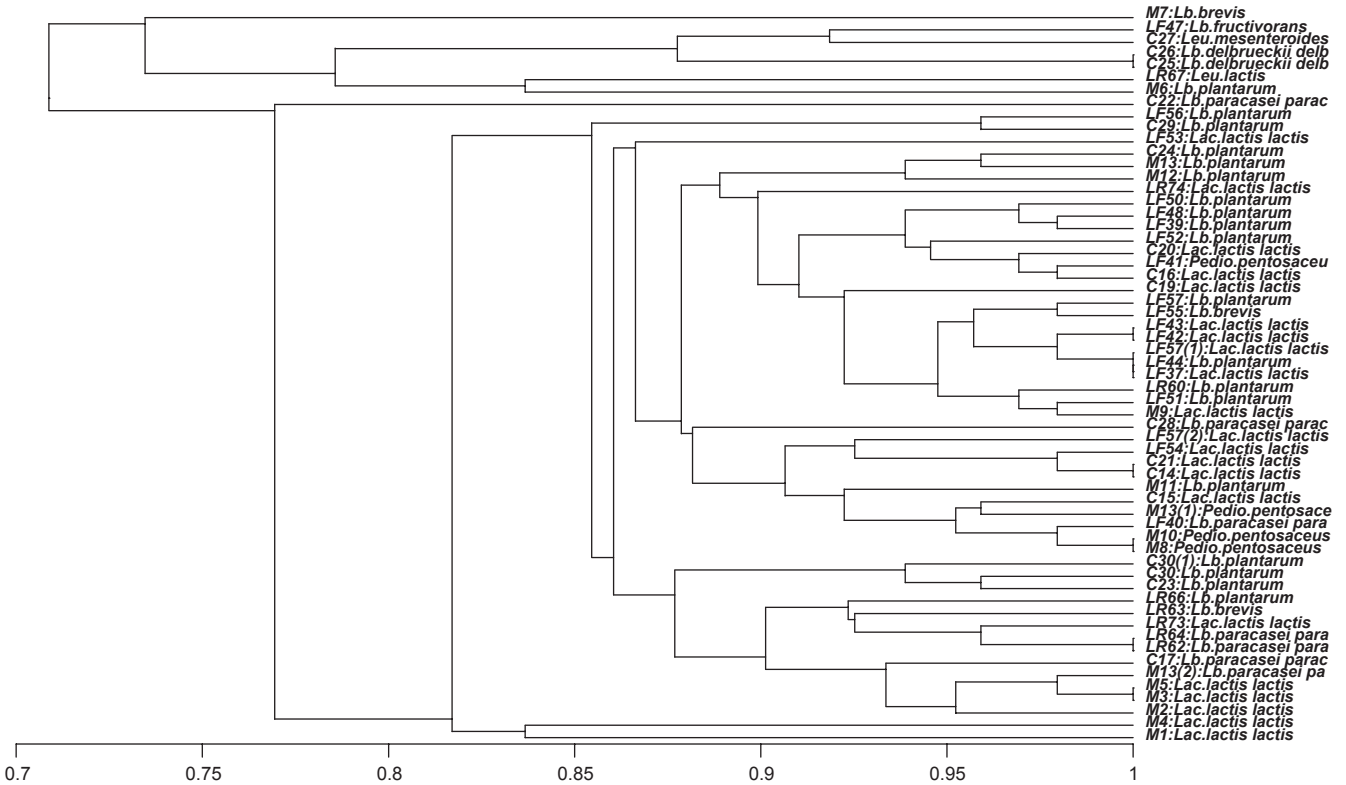


**Figure 3** Homology tree dendogram of the carbohydrate fermentation profiles obtained with the API 50-CHL system. The similarity matrix of the profiles was subjected to cluster analysis by the unweighted pair groups average linkage analysis clustering method (using the simple matching coefficient). Vertical lines of the dendogram represent the degree of similarity shared by the groups connected by the lines. Codes: M (Milk), C (Curd), LF (*Lighvan* Fresh cheese), LR (*Lighvan* Ripened cheese).

Table 7 shows the carbohydrate fermentation patterns of species from *Lighvan* cheese obtained with the API 50-CHL system. None of the isolates fermented erythritol, L-xylose, adonitol, methyl-β D-xylopyranoside, D-lyxose, D-fucose, L-fucose, D-arabitol and 2-ketogluconate. All isolates fermented D-glucose except *L. delbrueckii* ssp. *delbrueckii*. All strains fermented D-lactose except *L. delbrueckii* ssp. *delbrueckii* and *L. fructivorans*. In total, 51 different carbohydrate fermentation profiles were observed. The dendogram obtained after UPGMA analysis is shown in Fig. 3.

The importance of LAB in cheese production and ripening has been investigated by many scientists. Among them, lactococci plays an important role in the acidification of milk. Lactobacilli participate in the flavour improvement because of proteolytic and lipolytic activities. Leuconostoc species and *L. brevis* because of their heterofermentative property are responsible for gas production and subsequently results in making some holes in *Lighvan* cheese.

## CONCLUSION

Among ninety-five identified LAB isolated from four manufacturing stages, the highest number belonged to *E. faecium* (22.44%), *Lac. lactis* ssp. *lactis* (20.4%), *L. plantarum* (18.36%) and *E. faecalis* (14.28%). However, other species shared lower percentages. The predominant genera and species in each manufacturing stages were also identified. In milk stage, the predominant species were *Lac. lactis* ssp. *lactis* (26.8%), *E. faecalis* (21.73%) and *L. plantarum* (17.39%), while in ripening stage, the most abundant species were *E. faecium* (34.61%), *E. faecalis* (19.23%) and *Lac. lactis* ssp. *lactis* (15.38%). To apply these strains at the industrial scale, more attention should be paid to the identification of these species at subspecies level; this will require further accurate and precise molecular assays.

## ACKNOWLEDGEMENTS

The research reported herein was supported by funds from the Ministry of Mines and Industries as well as Razavi Dairy Industry. The authors gratefully acknowledge Mr. Pourakrami and Dr. Bazmi with regard to collecting cheese samples from *Lighvan* village.

## REFERENCES

Aymerich T, Holo H, Havarstein L S, Hugas M, Garriga M and Nes I F (1996) Biochemical and genetic characterization of enterocin A from

*Enterococcus faecium*, a new antilisterial bacteriocin in the pediocin family of bacteriocins. *Applied Environmental Microbiology* **62** 1676–1682.

Barouei J, Karbasi A and Ghoddusi H B (2008) Lactic Microflora present in *Lighvan* ewes' milk cheese. *International Journal of Food Properties* **11** 407–414.

Caridi A (2003) Identification and first characterization of lactic acid bacteria isolated from the artisanal ovine cheese Pecorino del Poro. *International Journal of Dairy Technology* **56** 105–110.

Centeno J A, Menéndez S, Hermida M and Rodriguez-Otero J L (1999) Effect of the addition of *Enterococcus faecalis* in Cebreiro cheese manufacture. *International Journal of Food Microbiology* **48** 97–111.

Devoyod J J (1969) Microbial flora of Roquefort cheese IV. Enterococci. *Lait* **49** 637–650.

Devoyod J J and Desmazeaud M (1971) Microbial associations in Roquefort cheese. III. Action of enterococci and lactose-fermenting yeasts on lactobacilli. *Lait* **51** 399–415.

Devoyod J J and Muller M (1969) Microbial flora of Roquefort cheese. III. Lactic streptococci and leuconostocs. Influence of various contaminating microorganisms. *Lait* **49** 369–399.

Farias M E, de Ruiz-Holgado A A P and Sesma F (1994) Bacteriocin production by lactic acid bacteria isolated from regional cheeses: inhibition of food borne pathogens. *Journal of Food Protection* **57** 1013–1015.

Foulquie Moreno M R, Sarantinopoulos P, Tsakalidou E and DeVuyst L (2006) The role and application of enterococci in food and health. *International Journal of Food Microbiology* **106** 1–24.

Fox P F, McSweeney P, Cogan T M and Guinee T P (2000) *Fundamentals of Cheese Science*, pp. 536–539. Gaithersburg, MD: Aspen Publishers.

Giraffa G (1995) Enterococci bacteriocins: their potential as anti-Listeria factors in dairy technology. *Food Microbiology* **12** 291–299.

Giraffa G (2003) Functionality of enterococci in dairy products. *International Journal of Food Microbiology* **88** 215–222.

Gurses M and Erdogan A (2006) Identification of lactic acid bacteria isolated from Tulum cheese during ripening period. *International Journal of Food Properties* **9** 551–557.

Joosten H M L J and Nunez M (1996) Prevention of histamines formation in cheese by bacteriocin producing lactic acid bacteria. *Applied Environmental Microbiology* **62** 1178–1181.

Kafili T, Razavi S H, Emam Djomeh Z, Naghavi M R, Álvarez-Martín P and Mayo B (2009) Microbial characterization of Iranian traditional *Lighvan* cheese over manufacturing and ripening via culturing and PCR-DGGE analysis: identification and typing of dominant lactobacilli. *European Food Research Technology* **229** 83–92.

Lopez-Diaz T M, Alonso C, Roman C, Garcia-Lopez M L and Moreno B (2000) Lactic acid bacteria isolated from a hand-made blue cheese. *Food Microbiology* **17** 23–32.

Marino M, Maifreni M and Rondinini G (2003) Microbiological characterization of artisanal Montasio cheese: analysis of its indigenous lactic acid bacteria. *FEMS Microbiology Letters* **229** 133–140.

Navidghasemizad S, Hesari J, Saris P and Nahaei M R (2009) Isolation of lactic acid bacteria from *Lighvan* cheese, a semi hard cheese made from raw sheep milk in Iran. *International Journal of Dairy Technology* **62** 260–264.

Salvadori B B (1969) Bitter flavor in blue cheeses. *Scienza e Tecnica Lattiero-casearia* **20** 1–14.

Sarantinopoulos P, Kalantzopoulos G and Tsakalidou E (2002) Effect of *Enterococcus faecium* on microbiological, physiochemical and sensory characteristics of Greek Feta cheese. *International Journal of Food Microbiology* **76** 93–105.