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Homofermentative lactic acid bacteria of a traditional cheese, Comlek peyniri from Cappadocia region

Cisem Bulut[1], Hatice Gunes[2], Burcu Okuklu[2], Sebnem Harsa[1], Sevda Kilic[3], Hatice Sevgi Coban[2] and Ali Fazil Yenidunya[2]*

[1] Izmir Institute of Technology, Biotechnology Programme, 35430 Gulbahce, Urla, Izmir, Turkey
[2] Department of Biology, Science Faculty, Izmir Institute of Technology, 35430 Gulbahce, Urla, Izmir, Turkey
[3] Ege University, Faculty of Agriculture, Department of Dairy Technologies, Bornova, Izmir, Turkey

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Comlek peyniri is a typical artisanal cheese in Central Anatolia. This type of cheese was made by using the indigenous lactic acid bacteria (LAB) flora of cow or ewes' milk. Majority of the samples were taken from fresh cheese because the aim was to isolate homofermentative LAB. Initially 661 microbial isolates were obtained from 17 cheese samples. Only 107 were found to be homofermentative LAB. These isolates were selected and identified by using both phenotypic and molecular methods. Phenotypic identification included curd formation from skim milk, catalase test, Gram staining and light microscopy, growth at different temperatures and salt concentrations, arginine hydrolysis, gas production from glucose, and carbohydrate fermentation. Molecular identification was based on the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) of the 16S rRNA gene-ITS (internally transcribed spacer) region. By combining the phenotypic and molecular identification results, isolates belonging to each of the following genera were determined at species or subspecies level: 54 *Lactococcus lactis* subsp. *lactis*, 21 *Enterococcus faecium*, 3 *Ec. faecalis*, 2 *Ec. durans*, 10 *Ec.* sp., 15 *Lactobacillus paracasei* subsp. *paracasei*, and 2 *Lb. casei* strains. Technological characterisation was also performed by culturing each of the strains in UHT skim milk, and by monitoring pH change and lactic acid production at certain time intervals through the 24 h incubation. Results of the technological characterisation indicated that 33% of the isolates (35 strains) were capable of lowering the pH of UHT milk below 5·3 after 6 h incubation at 30 °C. Thirty four of these strains were *Lc. lactis* subsp. *lactis*, and only one was an *Ec. faecium* strain.

Keywords: Identification, homofermentative lactic acid bacteria, 16S rRNA gene-ITS RFLP.

Traditional cheese fermentation is essentially based on the activity of indigenous LAB flora. Industrial production, on the other hand, requires defined starter cultures with desirable technological characteristics. Many of the commercial starter strains are homofermantative and they have been mostly selected and developed from *Lactococcus* species (Beresford et al. 2001). Starters initiate fermentation by producing lactic acid that results in curd formation. They also contribute to the maturation process by producing aromatic compounds via proteolytic activity. Furthermore, growth of certain pathogenic microorganisms can be inhibited or controlled by antimicrobial substances which are produced by the starter bacteria (Beresford et al. 2001).

*For correspondence; e-mail: afazilyenidunya@iyte.edu.tr

For the last two decades, Turkey has been experiencing an accelerating uncontrolled urbanisation. In parallel with this urbanisation, artisanal knowledge that propagates the traditional food making methodology through generations, disappears. This of course endangers the LAB microflora since little has been done to conserve the natural diversity of LAB.

Comlek peyniri is one type of traditional cheese and is very common in Central Anatolia. Although production recipes might change from one village to another, even among personal applications, the main steps of the process are as follows: clarification of raw milk, heating, cooling, the addition of rennet, incubation, pressing (whey expulsion), size reduction, salting, and finally ripening in earthen pots, which also serve as the packaging material. During the period of ripening, the pots are buried into volcanic

ashes, tuff, locally called 'kisir', in the upside down position, in natural caves specific to the region. Maturation process lasts between 3 and 6 months and the consumption time coincides with the winter season.

In the present work, PCR-RFLP pattern analysis of 16S rRNA gene-ITS clearly demonstrated that this method could differentiate strains of LAB at species level. Furthermore, the method also identified some diverged RFLP patterns within the strains of *Lactococcus lactis* subsp. *lactis*.

Technological characterisation of the isolated strains was carried out by measuring the acidifying activity and lactic acid production profiles (Cogan et al. 1997; Herreros et al. 2003).

The aim of this work was thus to characterize homofermentative LAB from a traditional Turkish cheese, Comlek peyniri, by using microbiological, molecular, and technological methods.

Material and Methods

Origin of samples and reference strains

In total 17 cheese samples were used for the isolation of LAB. Samples were obtained from the different stages of cheese making process. Reference strains were obtained from the Spanish Type Culture Collection (CECT) and Agricultural Research Service Culture collection (ARS, NRRL; Peoria, IL., USA): *Lc. lactis* subsp. *lactis* (CECT 4432), *Lc. lactis* subsp. *lactis* biovar. *diacetylactis* (CECT 4431), *Enterococcus faecium* (CECT 4102), *Ec. faecalis* (CECT 184), *Ec. gallinarum* (CECT 970), and *Lactobacillus casei* subsp. *casei* (NRRL B1922).

Isolation and growth conditions

Samples were analysed by the dilution pour plate method. Cheese samples (10 g) were homogenised in 90 ml sterile, a quarter strength Ringers solution. Decimal dilutions of the homogenates were pour-plated on specific media for different groups of LAB: M17 agar medium (Terzaghi et al. 1975), pH 7·15, for lactococci; MRS agar medium, pH 6·6 (De Man et al. 1960) for lactobacilli; and for enterococci, Slanetz and Bartley medium, pH 7·2. All the pour-plates were incubated at 30 °C for 3 d. For the initial selection of homofermentative LAB, randomly chosen colonies were cultured in reconstituted milk (RSM).

Phenotypic identification

Selection of isolates according to fermentative properties. Individual colonies were inoculated into 100 g RSM/l broths which were sterilised at 113 °C for 10 min. They were incubated at 30 °C for 24 h, and changes in the appearance of RSM were monitored. When curd formation was achieved, the incubation was terminated. Another 24 h incubation was allowed for those cultures which could not coagulate skim milk during the first 24 h. Thus, the isolates which were able to coagulate milk even after the 48 h incubation were also considered to be homofermentative LAB. Others were eliminated. Homofermentative isolates were then simple stained in order to observe cell shape and arrangements. Samples showing heterogeneous cell morphology were also eliminated. Consecutive subculturing was performed on the selected isolates. After Gram staining and catalase test, all Gram positive and catalase negative isolates were stored −80 °C in MRS broth containing 20% glycerol.

Physiological and biochemical identification. For the phenotypic identification of cocci shaped isolates the following tests were performed: gas production from glucose, growth at different temperatures (10, 40 and 45 °C), and different NaCl concentrations (20, 40 and 65 g/l), arginine hydrolysis, and gas production from citrate. For species and subspecies level identification carbohydrate fermentation tests were performed in 96-well plates by using the following 14 substrates: L(+)-arabinose, D(+)-galactose, lactose, maltose, D(+)-mannitol, raffinose, sucrose, D(−)-salicin, sorbitol, D(+)-trehalose, D(+)-xylose, glycerol, D(+)-mannose, and D(−)-ribose (Lopez-Diaz et al. 2000; De Urraza et al. 2000): cells from overnight cultures were pelleted by centrifugation at 9000 ***g*** for 10 min and were then washed and resuspended in MRS broth without glucose, containing bromocresol purple as the pH indicator. First, 40 µl filter sterilised (0·22 µm Millipore) sugar solutions (100 g/l) were pipetted into the each well, and 160 µl cell suspension were then added. Two control experiments included in each of the plates were glucose fermentation (positive control), and cell suspension without any sugar (negative control). All the fermentation reactions were prepared in duplicates. After 24 h incubation at 30 °C, the plates were read at 690 nm absorbance in an automated microtitre plate reader (Biotek Instruments Inc.). Sugar fermentation resulted in the change of the colour from purple to yellow, and turbidity was increased.

For the identification of rod shaped isolates gas production from glucose, growth at 15 and 45 °C, at 65 g NaCl/l concentration, arginine hydrolysis, and 14 carbohydrate fermentation tests were performed.

PCR-RFLP of 16S rRNA gene-ITS region

Genomic DNA was prepared by following the protocol of Cardinal et al. (1997) with some modifications. Cells from 10 ml overnight cultures in MRS broth were harvested by centrifugation at 3300 ***g*** for 5 min. The pelleted cells were suspended in 200 µl lysis buffer (250 g sucrose/l and 30 mg lysozyme/ml in 1 × TE, 10 mM-Tris–HCl, 1 mM-EDTA, pH 8), and incubated at 37 °C for 1 h. After the lysis, 370 µl proteinase K buffer (1 mg proteinase K/ml and 12·3 g SDS/l in 1 × TE, pH 8) were added. Samples were

further incubated at 37 °C for 10 min. Following deproteinization, 100 µl 5 M-NaCl and 80 µl CTAB/NaCl solutions (100 g cetyltrimethylammonium bromide/l and 0·7 M-NaCl) were added, and the samples were then incubated at 65 °C for 10 min. Chloroform extraction was performed twice using one sample volume (Chloroform/isoamyl alcohol: 24/1). DNA wool was obtained by the addition of one sample volume of isopropanol and washed in 500 µl, 70% ethanol. DNA was then pelleted, dried and dissolved in 100 µl RNase solution (100 µg RNase/ml in 1 × TE). After incubation at 37 °C for 1 h the volumes of the samples were adjusted to 400 µl with 1 × TE. DNA was solubilised by alternating heat shocks (at 80 °C for 10 min and at −20 °C for 20 min). After phenol/chloroform extraction, DNA was precipitated by adding 1/10 sample volume of 5 M-NaCl and 2 volumes of 99% ethanol. Pellets were washed in 70% ethanol. Finally DNA was solubilised in 1 × TE, and stored at −20 °C.

Amplification of 16S rRNA gene-ITS region was performed by using the following primers: Forward, 5′-AGAGTTTGATCCTGGCTCAG-3′ (Mora et al. 1998); and reverse, 5′-CAAGGCATCCACCGT-3′ (Jensen et al. 1993). The forward primer is complementary to the upstream of 16S rRNA gene of *Escherichia coli* K12 strain (Accession number: AE000452, nucleotides from 1 to 20), and the reverse is complementary to the upstream sequences of 23S rRNA gene of *Esch. coli* 278710 (Accession number: AJ278710, nucleotides from 18 to 32). Each of the polymerase chain reactions (PCR) was performed in a 50 µl reaction volume containing 50 ng genomic DNA as the template, 0·2 mM-dNTPs, 1·5 mM-$MgCl_2$, 10 pmol each of the DNA primers in 1 × PCR buffer (10 mM-Tris–HCl, 50 mM-KCl, 8 g Nonident P40/l, pH 8; MBI Fermentas, Lithuania), and 1·25 units *Taq* DNA polymerase (MBI Fermentas). Amplification conditions were as follows: an initial denaturation step of 5 min at 94 °C; 40 amplification cycles, each consisting of 1 min denaturation at 94 °C, 1 min annealing at 42 °C, and 1 min elongation at 72 °C, steps. Reactions were terminated with a final extension step for 10 min at 72 °C. PCR amplifications were performed in a Mini Cycler System (MJ Research INC., USA).

Two restriction endonucleases, *Hae*III and *Taq*I (MBI Fermentas), were used for the digestion of amplification products, differing in size from 1700 bp to 2000 bp. One fourth of the amplification products, approximately 200 ng, was digested with 10 units of each of the enzymes overnight. Before and after the digestion, DNA was extracted twice with chloroform and precipitated with ethanol (Sambrook et al. 1989).

Half of the digestion products were resolved in 2·5% agarose gel (Applichem, low EEO) by gel electrophoresis for 2·5 h at 60 mA in 1 × TAE (40 mM-Tris–acetate and 1 mM-EDTA, pH 8·0), and stained with ethidium bromide (1 µg/ml, final concentration). Images of the gels were recorded for further analysis in a gel documentation system (Vilber Lourmat).

Acidifying activity of the isolates

In order to determine acidifying activity, potantiometric (pH measurement) and titrimetric methods were used (Sagdıc et al. 2002; Xanthopoulous et al. 2001). Overnight cultures in MRS broths were inoculated into UHT skim milk at a 10 g/l inoculum level. Lactic acid production and decrease in pH were recorded after the 3, 6, 9, and 24 h incubation. Both of the experiments were repeated twice. The amount of lactic acid was determined by titrating 2 ml culture containing a few drops of the phenolphthalein indicator solution, against 0·1 M-NaOH until the first traces of pink colour appeared. Each ml 0·1 M-NaOH used for the titration was taken as equivalent to 9·008 mg lactic acid (AOAC, 1980).

Results

Phenotypic characterisation

Initially, 661 isolates were obtained and tested for the coagulation of skim milk broths. Among these, 246 isolates displayed better coagulation properties. The remaining isolates, which could not coagulate skim milk or produced textural defects such as gas bubbles, were eliminated. Some of the selected isolates, which displayed heterogeneous cell morphology or poor growth profiles, were also eliminated. After these selection steps, 90 cocci and 17 bacilli remained, and these were subjected to further identification procedures. The cocci isolates were differentiated into two genera, *Lactococcus* and *Enterococcus*, on the basis of growth at 10 °C, 45 °C and 65 g NaCl/l concentration. Arginine hydrolysis, citrate and sugar utilisation results indicated that all the lactococci isolates were *Lc. lactis* subsp. *lactis* (Teuber, 1995). Some of the *Lc. lactis* subsp. *lactis* isolates (A26, A30, A47, A48, B10, B20, B21, B28, C4, C15, C16, C28, C32, and C35, Table 1) showed atypical properties such as growth at 45 °C and 65 g NaCl/l concentration. Thus it was somewhat difficult to differentiate these isolates from enterococci.

Enterococcal isolates consisted of 36 strains. On the basis of sugar fermentation profiles 26 of these isolates could be differentiated into 3 species (Garvie, 1984; Devriese & Pot, 1995): 21 *Ec. faecium*, 3 *Ec. faecalis*, and 2 *Ec. durans* strains (Table 1). Arabinose, sorbitol and glycerol fermentation profiles enabled the discrimination of *Ec. faecium* strains from those of *Ec. faecalis*. *Ec. durans* strain was differentiated from the others because it could not ferment arabinose, mannitol, raffinose, sorbitol, trehalose, and xylose. Nevertheless, the remaining 10 enterococcal isolates could not be identified by the phenotypic methods (Table 1).

Twenty of the isolates were identified as lactobacilli since they were all Gram-positive, catalase-negative, and non-spore forming rod-shaped bacteria. Seventeen of the isolates could not produce CO_2 from glucose thus they

Table 1. Phenotypic and 16S rRNA gene-ITS RFLP haplotype grouping of the isolates

RFLP haplotype	Strains	Phenotypic identification	Number of isolates
HT1	*Lc. lactis* ssp *lactis* (CECT 4432), A1, A2, A3, A5, A6, A7, A8, A9, A10, A11, A12, A13, A14, A16, A19, A20, A21,A22, A23, A25, A26, A27, A28, A29, A30, A40, A44, A45, A46, A47, A48, B8, B11, B15, B20, B21, C1, C10, C11, C15, C16, C18, C19I, C19II, C22, C24, C32, C34, C35	*Lc. lactis* subsp. *lactis*	49
HT2	A35, A37, C4, C34	*Lc. lactis* subsp. *lactis*	4
HT3	C28	*Lc. lactis* subsp. *lactis*	1
HT4	A17, A18, A60, A61, A62, A63, A64, A65	*Enterococcus* sp.	8
H5	*Ec. faecium* (CECT 102), A31, A32, A33, A34, A38, A49, A50, B22, B23, B25, B26, B27, B28, B33, B34, C30, C31, C36	*Ec. faecium*	18
H6	A41	*Ec. faecium*	1
H7	A42, B24	*Ec. faecium*	2
T5	A43, B16	*Ec. durans*	2
T6	A56, B17	*Enterococcus* sp.	2
T7	*Ec. faecalis* (CECT 184), A39, A53, C38	*Ec. faecalis*	3
T8	C3, C5, C7, C8, C9, C12, C29, C37, D1, D3, D5, D6, D7, D8, D9	*Lb. paracasei* subsp. *paracasei*	15
T9	D2, D4	*Lb. casei*	2
Total			107

HT haplotype strains: Haplotypes, identified with both of the restriction enzymes, *Hae*III (H) and *Taq*I (T). H or T haplotype strains: Haplotypes, identified with only one of the restriction enzymes, *Hae*III (H) or *Taq*I (T). Reference strains showing similar RFLP patterns with some of the isolated strains, were also included into respective haplotype groups

were considered to be homofermentative and selected. All of the lactobacilli isolates could grow at 15 °C. Thus they were of mesophilic type. Carbohydrate fermentation profiles enabled the identification of 15 of the isolates as *Lb. paracasei* subsp. *paracasei* (Hammes & Vogel, 1995). Similarly 2 of the isolates, D2 and D4, were identified as *Lb. casei*.

PCR-RFLP pattern analysis of 16S rRNA gene-ITS region

Amplification products of 16S rRNA gene-ITS DNA from 107 isolates and 5 reference strains were digested with *Taq*I or *Hae*III. First, RFLP haplotypes specific to each of the restriction enzymes were identified by pooling the strains with identical restriction patterns, into different groups. Thus 16 RFLP groups, 7 *Hae*III (H-groups) and 9 *Taq*I (T-groups), were obtained. Four of these H- and T-groups contained identical strains. These groups were combined and re-named as HT-groups (HT1, HT2, HT3, and HT4, Table 1). Hence, in total 12 distinct RFLP haplotype groups were obtained from the restriction pattern analysis of 107 homofermentative strains (Table 1). One representative restriction pattern from each of these haplotype groups is shown in Fig. 1.

The HT haplotype groups included 58% of the isolates. Group HT1 contained the largest number of isolates (45·4%), together with the reference strain *Lc. lactis* subsp. *lactis* (CECT 4432). Five of the *Lc. lactis* subsp. *lactis* isolates on the other hand formed two distinct haplotype groups, HT2 and HT3 (Table 1).

Acidifying activity

Thirty four of the *Lc. lactis* subsp. *lactis* strains (A2, A3, A5, A6, A8, A9, A10, A11, A12, A13, A14, A20, A21, A22, A23, A25, A26, A27, A28, A30, A44, A45, A47, A48, B15, C1, C10, C11, C15, C18, C19I, C19II, C22, and C24) which were included into the HT1 haplotype group (Table 1), were found to be capable of lowering the pH of UHT milk below 5·3 after 6 h incubation at 30 °C (area 1, Fig. 2). Only one of the *Ec. faecium* strains (A31 of H5, Table 1) had similar acidifying activity.

Acidifying profiles of the 34 *Lc. lactis* subsp. *lactis* strains (62%) revealed that pH of UHT milk decreased sharply between 3 and 6 h, and after 9 h incubation, it reached almost a stationary phase, at pH 4·4 (area 1, Fig. 2). The profiles of remaining 20 lactococcal isolates were eliminated, because these profiles appeared to be overlapping with those of the enterococci and lactobacilli isolates, and making the diagram much more complicated. Enterococcal isolates did not seem to reach the same pH point even after 20 h incubation (area 2 and 2–3, representing acidifying profiles of 90% of the enterococcal isolates, Fig. 2). Lactobacilli strains displayed the slowest acidifying activity (area 2–3, and 3, representing 100% of the lactobacilli isolates, Fig. 2).

Lactic acid production profiles of the isolated strains were also analysed (data not shown). For the *Lc. lactis* subsp. *lactis* strains lactic acid production started to increase immediately after the third hour of incubation, and reached plateau values between 6 and 8 mg lactic acid/ml by the ninth h. The enterococcal strains, however,

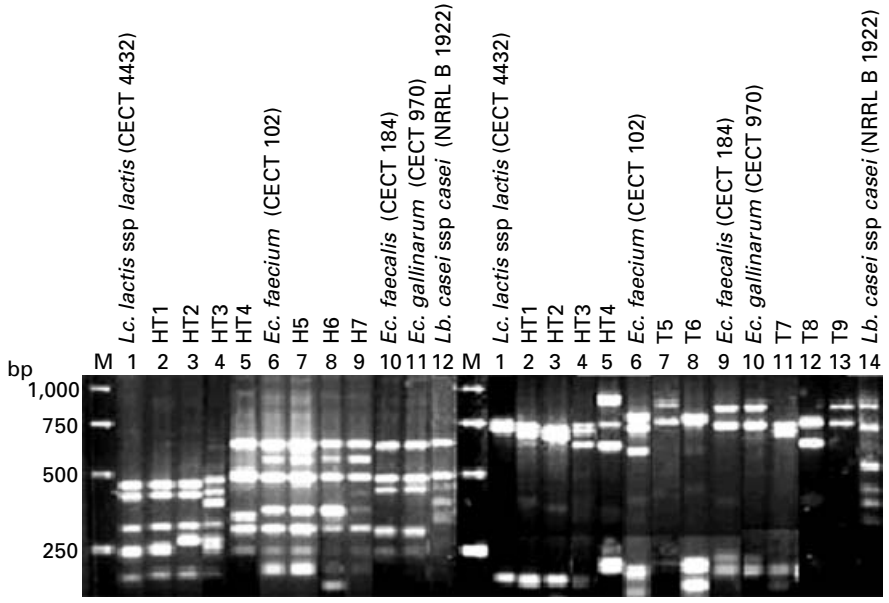

Fig. 1. PCR-RFLP profiles of the 12 haplotype groups. RFLP profiles of HT-haplotypes: *Hae*III profiles (left), lanes 2, 3, 4, and 5; *Taq*I profiles, lanes 2, 3, 4, and 5 (right). H-haplotypes, lanes 7, 8, and 9 (left). T-haplotypes, lanes 7, 8, 11, 12, and 13 (right). RFLP profiles of the five reference strains: with *Hae*III, lanes 1, 6, 10, 11, and 12 (left); with *Taq*I, lanes 1, 6, 9, 10, and 14 (right). Names of the RFLP haplotype groups and reference strains were indicated above the respective lanes. M was a DNA size marker (1 kb GeneRuler, MBI Fermentas).

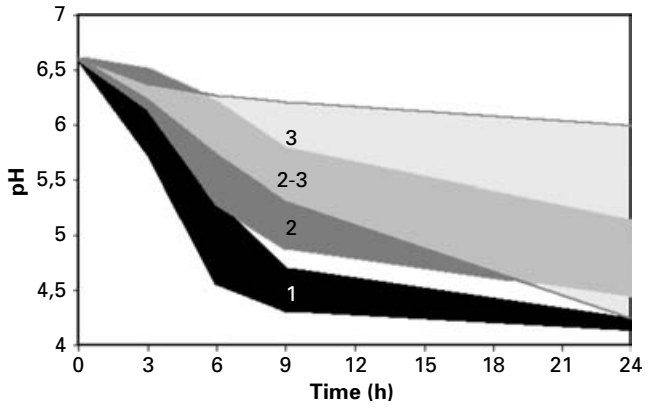

Fig. 2. Acidifying profiles of the isolated strains after 0, 3, 6, 9, and 24 h of incubation in UHT skim milk at 30 °C: lactococcal profiles, area 1 (black); enterococcal profiles, areas 2 (dark grey) and 2–3; and profiles of lactobacilli, areas 2–3 and 3 (light grey).

produced much less lactic acid, ranging from 4 to 6 mg/ml, at the same incubation period. For the lactobacilli isolates almost linear profiles were obtained until the end of 24 h incubation.

Discussion

Lc. lactis subsp. *lactis* is considered to be the most important *Lactococcus* species in cheese fermentation (López-Díaz et al. 2000). The abundance of *Lc. lactis* subsp. *lactis* in different types of cheese has been reported (Ayad et al. 2001; Delgado & Mayo, 2003). A good acid producing starter LAB culture reduces the pH of milk from its normal value of 6·6 to 5·3 within 6 h incubation at 30 °C (Herreros et al. 2003). Technological characterisation results revealed unambiguously that 34 of the isolated lactococcal strains were able to lower the pH of UHT milk below 5·3 under such conditions. Twenty nine of these strains were isolated from a 4 day-old cheese sample, made of cows' milk, and 5 of them were from two, one day old, cheese samples, made of ewes' milk.

Fourteen of the lactococcal isolates showed atypical physiological properties, for example, growth in 65 g NaCl/l and at 45 °C. These features can be advantageous for the preparation of certain types of fermentation products. In a recent work, lactococcal isolates having similar atypical characteristics have also been reported (Fortina et al. 2003).

Identification of the lactococcal isolates was quite straightforward since there was a good correlation between the results of biochemical characterisation and of 16S rRNA gene-ITS RFLP pattern analysis. Both *Hae*III and *Taq*I digestion profiles could easily differentiate these isolates (HT1, Table 1) except 5 strains which displayed diverged RFLP patterns (strains of HT2 and HT3, Table 1).

Enterococci, have been known to be slow acid producers (Sarantinopoulos et al. 2001). In agreement with the literature, only one enterococcal isolate, A31 (H1, Table 1), was found to lower the pH below 5·5 after 6 h incubation.

The presence of enterococci in cheese has been questioned because they are known as the cause of some serious health problems, such as endocarditis and urinary tract infection (Beresford et al. 2001). Numbers of enterecocci in Mediterranean type of cheese for example can be as high as 10^6 cfu/g, in curds, and 10^7 cfu/g in the fully ripened cheese (Franz et al. 2003). They have on the other hand beneficial effects on the flavour development in many types of cheese (Lopéz-diaz et al. 2000). Eight of the enterococcal isolates (A39, A43, A53, A56, A60, B33, C30, C38; Table 1), for example, could produce gas from citrate. The breakdown of citrate results in the production of carbon dioxide and also some flavour compounds such as diacetyl, acetaldehyde, and acetoin. Besides bacteriocin production ability of enterococci is also beneficial in controlling the growth of some food pathogens (Sarantinopoulos et al. 2001). Thus, for such characteristics and together with other desirable technological and metabolic properties, enterococci have also been suggested as part of the defined starter cultures for different European cheeses (Franz et al. 2003).

Some of the enterococcal isolates could not be identified by the phenotypical characterisation methods. Similar problems have also been reported by others (Devriese & Pot, 1995; Andrighetto et al. 2001). On the other hand, 16S rRNA gene-ITS RFLP pattern analysis, especially with *Hae*III restriction enzyme, was found to be useful for the identification of most of the enterococcal isolates.

Lactobacilli are mostly involved in the ripening period of cheese manufacturing and they are rarely used as starters (Stiles & Holzapfel, 1997). In agreement with these reports, none of the lactobacilli isolates seemed to reduce the pH below 5·7 in 6 h.

Phenotypic characterisation results suggested that 15 of the lactobacilli isolates could be identified as *Lb. paracesei* subsp. *paracasei*, and two isolate, D2 and D4, as *Lb. casei* (Table 1). In accordance with these results, 16S rRNA gene-ITS RFLP profiles also divided these isolates into two RFLP haplotype groups, T4 and T5 (Table 1). However, a comparative identification of the lactobacilli isolates, using relevant reference strains, is still required.

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