Phenotypic and genetic diversity of enterococci isolated from Italian cheeses

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Summary. In the present study, 124 enterococcal strains, isolated from traditional Italian cow, goat and buffalo cheeses, were characterized using phenotypic features and randomly amplified polymorphic DNA polymerase chain reaction (RAPD–PCR). The RAPD–PCR profiles obtained with four primers and five different amplification conditions were compared by numerical analysis and allowed an inter- and intraspecific differentiation of the isolates. Whole-cell protein analysis by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was used as a reference method for species identification. The strains were identified as *Enterococcus faecalis* (82 strains), *E. faecium* (27 strains), *E. durans* (nine strains), *E. gallinarum* (four strains) and *E. hirae* (two strains). Species recognition by means of RAPD–PCR was in agreement with the SDS–PAGE results except for eight strains of *E. faecium* that clustered in separated groups. On the other hand, phenotypic identification based on carbohydrate fermentation profiles, using the rapid ID 32 STREP galleries, gave different results from SDS–PAGE in 12·1% of the cases. The majority of the strains had weak acidifying and proteolytic activities in milk. One *E. faecium* strain showed *van*A (vancomycin resistance) genotype while four strains showed a β-haemolytic reaction on human blood. Several strains showed antagonistic activity towards indicator strains of *Listeria innocua*, *Clostridium tyrobutyricum* and *Propionibacterium freudenreichii* subsp. *shermanii*.

Keywords: Phenotype, genotype, enterococci, Italian cheeses.

Enterococci are ubiquitous bacteria that can be found in plants and insects, and are normally present in the digestive tract of humans and animals. They can occur in several cheeses made with both pasteurized and raw milk, and in natural starter cultures used in the manufacture of traditional cheeses. They have also been

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proposed as part of defined starter cultures for different European cheeses such as Water-Buffalo Mozzarella (Villani & Coppola, 1994), Feta (Litopoulou-Tzanetaki *et al.* 1993), Venaco (Casalta & Zennaro, 1997) and Cebreiro (Centeno *et al.* 1999). Depending on the stage of ripening, enterococci can reach numbers of up to 10^6–10^8 CFU/g (Fontecha *et al.* 1990; Basso *et al.* 1994) due to their high tolerance to salt and acid. The metabolic and technological traits of many strains belonging to *Enterococcus faecalis*, *E. faecium* and *E. durans*, the species most frequently found in dairy products, are considered essential in determining the typical cheese taste and structure. The high proteolytic activity shown by some strains belonging to the species *E. faecalis* could contribute to the sensorial and textural properties of cheese, as reported by Centeno *et al.* (1999). Furthermore, enterococci are producers of esterases, which can play an important role in flavour formation (Tsakalidou *et al.* 1993), and of components which contribute to cheese flavour, such as diacetyl and acetoin (Centeno *et al.* 1996).

Some enterococcal strains have characteristics which can improve human or animal health. A strain of *E. faecium*, SF68, has been evaluated as a probiotic in several studies for its positive effects against diarrhoea in man and pigs (Underdahl, 1983), while other strains of *E. faecium* and *E. gallinarum* were found to inhibit the growth of *Clostridium* spp. and *Listeria* spp. when added to chicken feed (Scanlan *et al.* 1991).

In contrast with these positive features, enterococci are considered as emerging pathogens of humans and are often associated with hospital-acquired infections, such as endocarditis, bacteraemia, urinary tract, intra-abdominal and pelvic infections (Franz *et al.* 1999), with *E. faecalis* being responsible for the major part (Low *et al.* 1994). A reason for the rise of nosocomial infections related to enterococci might be their ability to develop resistance against most antibiotics currently used, with vancomycin resistance being one of the most recent acquisitions besides their intrinsic resistance against antibiotics such as β-lactams (French, 1998; Teuber *et al.* 1999). This, in combination with known virulence factors such as production of aggregation substances, gelatinase and haemolysin, determines that enterococci are not considered GRAS (generally recognized as safe) microorganisms.

A reliable identification of enterococci to the species and strain level is of great importance not only for hospital infections, because of the naturally occurring differences in susceptibility to antibiotics of the different species, or for epidemiological surveillance, but also for the dairy industry since the different species, as well as the different strains within the species, might have different biochemical traits which may influence the characteristics and quality of the end-product. Unfortunately, it has been impossible to find phenotypic characteristics that can be used to distinguish enterococci unequivocally from other genera of lactic acid bacteria (Leclerc *et al.* 1996).

Pulsed field gel electrophoresis (PFGE) of genomic macrorestriction fragments and restriction enzyme analysis (REA) of total chromosomal DNA are good identification methods, but are time- and money-consuming. An alternative for these methods could be the randomly amplified polymorphic DNA polymerase chain reaction (RAPD–PCR). Recently, Quednau *et al.* (1998) reported RAPD–PCR as a fast and reliable method for the identification of most *Enterococcus* spp. of clinical significance.

The aim of the present study was to identify the enterococci present in different Italian cheeses at species and intra-species level and to investigate some technologically relevant characteristics in order to improve the understanding of the significance

and role of these microorganisms in milk and dairy products. Potential pathogenic factors such as haemolysis on human blood, and vancomycin and teicoplanin resistance were also considered.

MATERIALS AND METHODS

Bacterial strains and growth conditions

The 124 enterococcal strains considered in this study were either from existing collections (isolated before 1997) or new isolates (1997) from Italian fresh and ripened cheeses at different stages of ripening and from milk and natural cultures used for cheese manufacture, chosen to give a good geographical spread. Traditional as well as PDO (protected denomination of origin) cheeses, produced with raw or pasteurized milk, depending on the type of cheese and dairy plant, were considered (Table 1). Cheese samples were homogenized in a solution of sodium citrate (20 g/l) using a Stomacher Lab Blender (PBI International, Milano, Italy), adequately diluted in $\frac{1}{4}$ strength sterile Ringer solution and spread on kanamycin aesculine azide (KAA; Oxoid, Garbagnate Milanese, Italy) plates. After 24 h incubation at 37 °C in aerobic conditions, colonies that displayed the typical enterococcal growth and cell morphology were picked up and purified on KAA plates, after which they were stored at −80 °C. All isolates investigated are FAIR-E strains of the FAIR-E collection (Vancanneyt *et al.* 1999). A complete list of the 124 strain numbers can be obtained from the latter catalogue by selecting all strains originating from Italian cheeses, milk and natural cultures, with the exception of the FAIR-E strains 61, 382 to 389, 392, 393, 404 and 405, which were not considered in the present study. FAIR-E 84 and 315, not listed in the catalogue, were also included and originate from soft cheese and mozzarella cheese respectively. Catalogue and strains are available at BCCM/LMG Bacteria Collection, Laboratory of Microbiology, University of Gent, Ledeganckstraat 35, B-9000 Gent, Belgium. The following type and reference strains were also obtained from the BCCM/LMG Bacteria Collection: *Enterococcus hirae* LMG 6399^T, *E. faecalis* LMG 7937^T, *E. casseliflavus* LMG 10745^T, *E. durans* LMG 10746^T, *E. faecium* LMG 11423^T, *E. gallinarum* LMG 13129^T. Indicator strains *Listeria innocua* LMG 11387^T, *L. innocua* LMG 13568, *Clostridium tyrobutyricum* LMG 1285^T and *Propionibacterium freudenreichii* subsp. *shermanii* LMG 16424^T were used as target strains in antagonistic tests. The two *L. innocua* strains were grown in brain–heart infusion broth (BHI, Oxoid), the *Clostridium* strain in reinforced clostridium medium (RCM, Oxoid) and the *Propionibacterium* strain in yeast extract–lactate (YEL) medium containing 21 g sodium lactate + 10 g tryptone + 10 g yeast extract + 250 mg K_2HPO_4 + 50 mg $MnSO_4$ per litre.

Phenotypic identification

The following biochemical–physiological characteristics were evaluated: catalase activity, growth in MRS broth at 10 and 45 °C, growth at pH 9·6, growth in MRS broth containing 65 g NaCl/l, and CO_2 production from glucose. The isolates were phenotypically identified with the rapid ID 32 STREP system according to the protocol specified by the manufacturer (bioMérieux Italia, Roma, Italy).

SDS–PAGE of cellular proteins

Whole-cell protein extracts were prepared and SDS–PAGE analysis was performed as described by Pot *et al.* (1994). The digitized and normalized patterns were added to the existing database of SDS–PAGE protein patterns of lactic acid

Table 1. *Origin of the enterococcal strains studied and species distribution on the basis of SDS–PAGE analysis of whole-cell proteins*

Cheese	Type of milk	Geographical area	Number of isolates	Species distribution
Asiago*	cow	North-East (Veneto)	20	13 *E. faecalis* 6 *E. faecium* 1 *E. hirae*
Montasio*	cow	North-East (Veneto, Friuli)	16	10 *E. faecalis* 3 *E. faecium* 2 *E. durans* 1 *E. gallinarum*
Monte Veronese*	cow	North-East (Veneto)	21	17 *E. faecalis* 3 *E. faecium* 1 *E. durans*
Fontina*	cow	North-West (Valle d'Aosta)	17	11 *E. faecalis* 4 *E. faecium* 2 *E. durans*
Mozzarella*	buffalo	South (Campania)	8	6 *E. faecalis* 2 *E. gallinarum*
Caprino	goat	South (Basilicata)	7	4 *E. faecalis* 1 *E. hirae* 1 *E. durans* 1 *E. gallinarum*
Scamorza	cow	North-East (Veneto)	5	1 *E. faecalis* 4 *E. faecium*
Others	cow	North (Veneto, Lombardia)	30	20 *E. faecalis* 7 *E. faecium* 3 *E. durans*

* Protected denomination of origin cheese.

bacteria at the BCCM/LMG Bacteria Collection (Pot & Janssens, 1993). Pattern storage and comparison was done on an MS-DOS compatible PC using GelCompar version 4.2 (Applied Maths, Kortrijk, Belgium).

RAPD–PCR analysis

For DNA extraction, strains were grown overnight in MRS broth. DNA was extracted with the guanidium extraction method of Pitcher *et al.* (1989). Amplification reactions were performed according to the protocols routinely adopted by the laboratories involved in the present work using the following primers and amplification conditions:

1. M13: 5′ GAGGGTGGCGGTTCT 3′ (Huey & Hall, 1989); 35 cycles of: 94 °C for 1 min, 40 °C for 20 s, ramp to 72 °C at 0·6 °C s^{-1}, 72 °C for 2 min.
2. D8635: 5′ GAGCGGCCAAAGGGAGCAGAC 3′ (Akopyanz *et al.* 1992). Two different amplification reactions were applied: (i) an initial step of 94 °C for 2 min followed by 35 cycles of: 94 °C for 1 min, 42 °C for 1 min, 72 °C for 1·5 min; a final step at 72 °C for 10 min and (ii) one pre-PCR cycle of 94 °C for 5 min, 40 °C for 5 min and 72 °C for 5 min followed by 35 cycles of: 94 °C for 1 min, 52 °C for 1 min, 72 °C for 2 min; a final step at 72 °C for 10 min.
3. AP4: 5′ TCACGCTGCA 3′ (Barbier *et al.* 1996). An initial step of 94 °C for 2 min followed by 45 cycles of: 94 °C for 30 sec, 35 °C for 1 min, 72 °C for 2 min; a final step at 72 °C for 5 min.

4. 1253: 5′ GTTTCCGCCC 3′ (Akopyanz *et al.* 1992). One pre-PCR cycle of 94 °C for 5 min, 36 °C for 5 min and 72 °C for 5 min, followed by 30 cycles of: 94 °C for 1 min, 36 °C for 1 min, 72 °C for 2 min; a final step at 72 °C for 10 min.

Amplification products were separated by electrophoresis on agarose gel (15 g/l) in 0·5 × TBE buffer (0·45 mM-Tris–HCl, 0·45 mM-boric acid, 1 mM-EDTA, pH 8·3). The gels were stained in ethidium bromide and photographed on a u.v. trans-illuminator. Photo-positives were scanned into a computer and subsequently analysed using the GelCompar software version 4.1 (Applied Maths, Kortrijk, Belgium). Grouping of the RAPD–PCR patterns was performed by means of the Pearson product moment correlation coefficient and the unweighted pair group method using arithmetic averages (UPGMA) cluster analysis.

Minimal inhibitory concentration (MIC)

Strains were inoculated in Trypticase Soy Broth (Oxoid) with 3 g yeast extract/l and with either vancomycin or teicoplanin at a concentration range from 2 to 64 μg/ml. After an overnight incubation at 37 °C, growth was noted and the MIC (the first dilution at which no growth was observed) was determined.

Detection of glycopeptide resistance genotypes

The presence of *van* genes was checked using PCR as described by Dutka-Malen *et al.* (1995). Four different primer pairs (*van*A, *van*B, *van*C1 and *van*C2) were used in the assay.

Haemolysis

Blood haemolysis was evaluated on Columbia agar plates (Bracco–Merck, Milano, Italy) supplemented with 5% human blood and incubated at 37 °C for 24 h.

Antimicrobial testing

Enterococci cultured for 16 h were checked for inhibitory activity by the agar well diffusion assay or the agar spot test. Each of the indicator strains was grown in the corresponding medium until it reached an optical density of approximately 0·45 at 600 nm. To detect inhibitory activity towards *C. tyrobutyricum* LMG 1285^T, plates were prepared by adding 430 μl culture to 15 ml reinforced clostridium medium (RCM) with 10 g agar/l and wells with a diameter of approximately 5 mm were made, in which 25 μl supernatant of the enterococcal culture, obtained after three steps of centrifugation at 15800 ***g*** for 15 min, were pipetted. Plates were incubated anaerobically at 37 °C for 24 h. To detect inhibitory activity towards *P. freudenreichii* subsp. *shermanii* LMG 16424^T and both strains of *L. innocua*, the agar spot-test was used. For this method, 3·5 ml of top agar medium (with 7 g agar/l) inoculated with 100 μl indicator strain culture were poured on 20 ml bottom agar from the appropriate medium (with 15 g agar/l). Cell-free supernatant (10 μl) was spotted on the plates, which were incubated aerobically at 30 °C for 24 h. For both methods the diameter of inhibition haloes was noted.

Acidifying and proteolytic activities

The acidifying activity in milk was tested by inoculating 10 ml pasteurized milk (80 °C for 30 min) with an overnight culture (100 μl), which had been previously transferred twice in pasteurized milk. After 6 and 16 h of incubation at 37 °C, coagulation was noted and the pH was measured.

Proteolytic activity was tested by plating the isolates on plates made with skimmed milk powder (100 g/l) and agar (15 g/l). After overnight incubation at 37 °C in aerobic conditions, the plates were viewed for clear zones around single colonies.

Biogenic amine production

Biogenic amine production was evaluated in the decarboxylase agar medium described by Joosten & Northolt (1989) containing tyrosine, histidine, lysine or ornithine (each 20 g/l) as precursors. Then, 1 μl enterococcal culture transferred twice in MRS containing 1 g of the appropriate amino acids + 1 mg of pyridoxal 5′-phosphate/l was spotted on the decarboxylase agar medium and incubated anaerobically at 37 °C for 72 h.

Production of volatile compounds in milk

Enterococcal cultures were prepared from an overnight MRS culture and two subcultures in UHT milk supplemented with 3 g yeast extract/l. The cultures were then inoculated (1 %, v/v) in UHT milk without yeast extract and incubated at 37 °C for 24 h. The samples for the static headspace gas chromatographic analysis (HS–HRGC) were prepared in 10 ml vials containing 2 ml culture, 1 g NaCl, and 10 μl methylpropylketone (1000 ppm) as internal standard. HS–HRGC analysis was performed by means of an HS 250 autosampler (Carlo Erba Instruments, Milano, Italy) and an HRGC 5300 (Carlo Erba Instruments) modified with a cryofocusing unit (−140 °C); a 50 m (0·32 mm i.d.) capillary column with a 3 μm polydimethylsiloxan coating was used. Head space (2 ml) was injected, the column temperature was held at 40 °C for 3 min and then ramped to 180 °C at 5 deg C/min. Helium was used as carrier gas. Peak detection was obtained by means of a flame-ionization detector (FID, Carlo Erba Instruments) integrated with an SP4270 integrator (Spectra Physics, Carlo Erba Instruments).

RESULTS

Phenotypic identification of enterococcal species

The isolates were initially assigned to the genus *Enterococcus* on the basis of phenotypic characterization. Nearly all the isolates possessed the phenotypic traits that are commonly used to distinguish enterococci from other Gram-positive, facultatively anaerobic cocci, i.e. ability to grow in the presence of 65 g NaCl/l, at pH 9·6 and both at 10 and 45 °C. Only one strain was unable to grow in the presence of 65 g NaCl/l, while growth at 10 °C was not observed in 18 out of the 124 cheese isolates.

Identification of strains was performed with the rapid ID 32 STREP system and the SDS–PAGE analysis of whole-cell proteins. Most of the strains were allotted to the species *E. faecalis*, *E. durans* and *E. hirae* by both methods, and biochemical identification levels varied from good to excellent. However, 33 % of isolates identified as *E. faecium* with SDS–PAGE were not assigned to this species by the rapid ID 32 STREP system, and the identification of a further 37 % was considered doubtful or unacceptable due to atypical fermentation reactions in the different galleries. Moreover, five isolates recognized as *E. casseliflavus* by the rapid ID 32 STREP system were assigned to other species (*E. gallinarum*, *E. faecalis* and *E. faecium*) by SDS–PAGE.

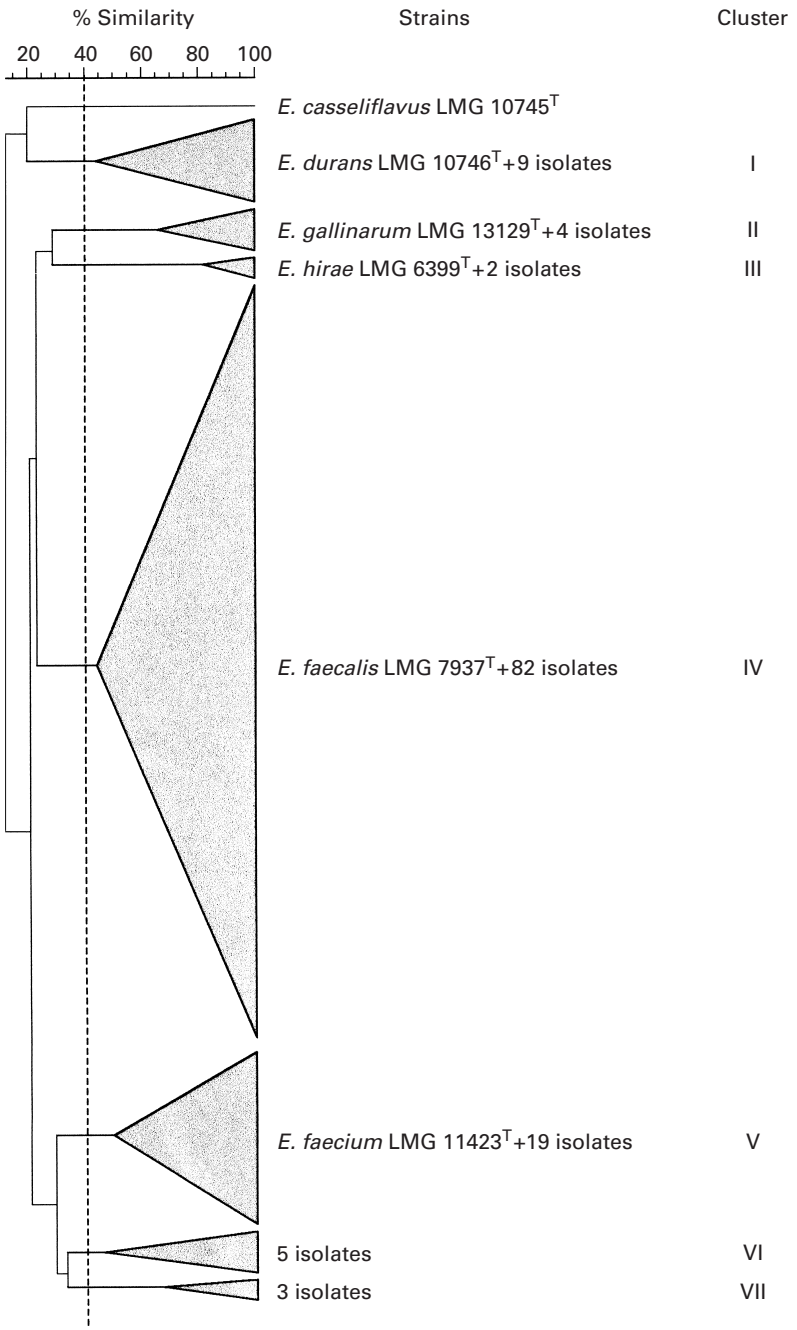

Fig. 1. UPGMA-based dendrogram from the randomly amplified polymorphic DNA polymerase chain reaction profiles of the enterococcal isolates and the type strains.

As the correlation between SDS–PAGE of whole-cell protein pattern similarity levels and DNA–DNA hybridization values are well-documented also for enterococci (Descheemaeker *et al.* 1997), SDS–PAGE was chosen as reference method for identification at the species level. The numerical analysis of SDS–PAGE profiles assigned 66·2% of the cheese isolates to the species *E. faecalis*, 21·8% to *E. faecium*, 7·2% to *E. durans*, 3·2% to *E. gallinarum* and 1·6% to *E. hirae*.

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RAPD–PCR analysis

Four primers and five amplification conditions were tested in the RAPD–PCR analysis; the use of two different amplification cycles for primer D8635 allowed a higher discrimination at the strain level. The value for the reproducibility of the RAPD–PCR assay, DNA extraction and running conditions, evaluated by analysis of repeated DNA extracts of the type strains, was greater than 91%. After a numerical analysis of the combined RAPD–PCR patterns of the 124 enterococcal strains studied, seven clusters and one single strain were obtained at a similarity level of 40%, arbitrarily chosen for defining species (Fig. 1). Cluster I grouped *E. durans* LMG 10746^T as well as nine isolates originating from different cheeses and geographical areas. Cluster II contained *E. gallinarum* LMG 13129^T and four cheese isolates, while cluster III grouped *E. hirae* LMG 6399^T and two cheese isolates. Most of the cheese isolates (82 strains, 66·2%) were found in cluster IV together with *E. faecalis* LMG 7937^T. Cluster V contained *E. faecium* LMG 11423^T and 19 cheese isolates while clusters VI and VII consisted of five and three isolates respectively. When these latter isolates were studied by the species-specific primers of Dukta-Malen *et al.* (1995) and by SDS–PAGE of whole-cell proteins, they were all unequivocally assigned to the species *E. faecium*. No cheese isolate grouped together with the type strain of *E. casseliflavus*.

RAPD–PCR results agreed well with SDS–PAGE except for eight strains belonging to the *E. faecium* species. These strains were clustered by RAPD–PCR in two separate groups (VI and VII) close to cluster V containing the type strain of this species. The combined use of four primers and five amplification conditions allowed the detection of intra-specific differences (Fig. 2). The different cheese origins were not reflected in the dendrogram, just as strains isolated from different geographic areas did not form separated clusters.

Antibiotic sensitivity and blood haemolysis

MIC values of vancomycin and teicoplanin revealed that most of the isolates were susceptible to these antibiotics. As reported in Table 2, six *E. faecalis* and four *E. gallinarum* strains showed a low-level resistance to vancomycin (MIC values from 8 to 16 μg/ml). When DNA from these strains was used as template in the specific PCR with the primers described by Dutka-Malen *et al.* (1995), the *E. gallinarum* strains had the *van*C1 genotype while none of those considered van genes (*van*A, *van*B, *van*C1 and *van*C2) was found in the *E. faecalis* strains. Recently, a new type of low-level vancomycin resistance (*van*E, MIC = 16 μg/ml) was detected in one strain of *E. faecalis* (Fines *et al.* 1999); further studies on the presence of this gene in the *E. faecalis* cheese isolates showing a low vancomycin resistance phenotype will be necessary. A high-level resistance to both vancomycin and teicoplanin was found for one strain of *E. faecium* and the fragment of about 730 bp corresponding to a specific part of the *van*A gene was detected.

Haemolysin production was found at a very low frequency: indeed, only four strains of *E. faecalis* showed a slightly positive β-reaction on human blood (FAIR-E 74, FAIR-E 90, FAIR-E 92 and FAIR-E 372), while no α-haemolytic strain was found among the cheese isolates.

Behaviour in milk

Proteolysis in milk was detected in 24·2% of the strains, all identified as *E. faecalis*. Acid production in milk was poor, with only 20·2% of the strains showing

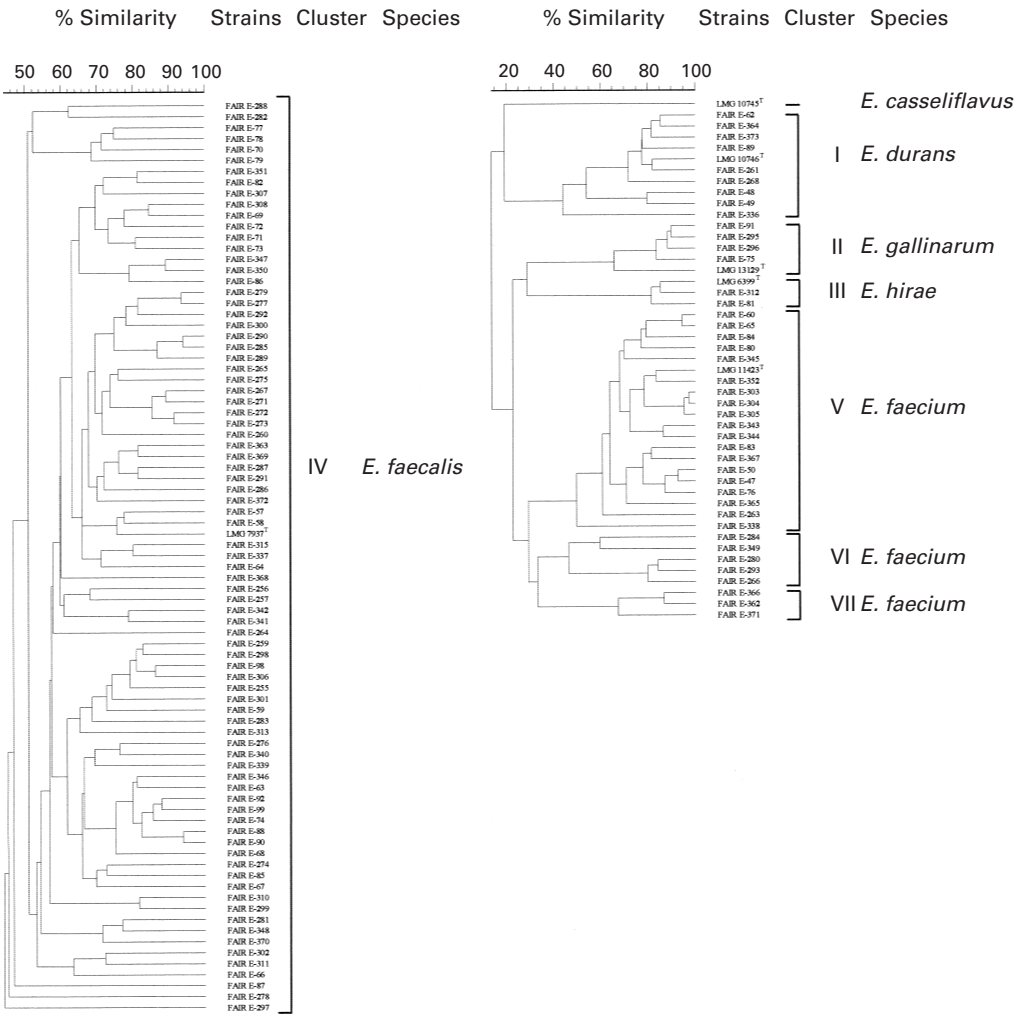

Fig. 2. Genetic diversity among the 124 enterococcal strains on the basis of the UPGMA dendrogram derived from the randomly amplified polymorphic DNA polymerase chain reaction combined patterns.

a pH less than 5·2 after 16 h of incubation at 37 °C. All but three of the strains with the highest acidifying activity showed proteolytic activity on milk plates, suggesting a correlation between the two traits.

After 6 h, 21 % of the strains, all identified as *E. faecalis*, were able to coagulate milk at a pH ranging from 5·8 to 6·2. Of these strains, all except one also showed proteolytic activity. In addition, there were five other strains (4 %) which possessed proteolytic activity, but did not coagulate milk after 6 h of incubation.

Volatile compounds production

Production of volatile compounds in milk was tested on 44 out of the 124 dairy isolates considered in the present study. Three major compounds were detected: acetaldehyde, acetoin and ethanol. Acetaldehyde production was variable and generally low; only 13·6 % of the strains, all belonging to the species *E. faecalis*, were able to produce more than 10 ppm of acetaldehyde after 24 h of incubation at 37 °C. Acetoin was detected in all but two of the samples and its concentration was always

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Table 2. *Minimal inhibitory concentration (MIC) values of vancomycin and teicoplanin and presence of* van *genes for some enterococcal strains isolated from Italian cheeses*

Species	Strains (FAIR-E)	MIC (μg/ml) vancomycin	MIC (μg/ml) teicoplanin	*van* genes†
E. faecalis	255, 257, 267, 301, 370	8	2	nd
	273	16	2	nd
E. gallinarum	75, 91	16	2	*van*C1
	295, 296	8	2	*van*C1
E. faecium	84	> 64	> 64	*van*A

nd, no *van* gene detected.
† The presence of *van* genes was detected with the primers described by Dutka-Malen *et al.* (1995).

Table 3. *Inhibitory effect of enterococcal isolates against four indicator strains*

Species	Strain number (FAIR-E)	*L. innocua* LMG 11387^T	*L. innocua* LMG 13568	*C. tyrobutyricum* LMG 1285^T	*P. freudenreichii* subsp. *shermanii* LMG 16424^T
E. faecium	76, 266	−	−	−	+
	84, 362	+	+	−	−
	366	−	+	−	−
E. faecalis	77, 92, 256, 257	−	−	+	+
	88, 90, 260, 298, 299	−	−	+	−
	255, 259	−	+	+	+

− No inhibitory effect.
+ Inhibition of growth.

below 90 ppm, except for *E. faecium* FAIR-E 345 which was able to produce up to 156·6 ppm of this compound. Of the strains tested, 41 % produced low amounts of ethanol (< 20 ppm), while a high production of ethanol ranging from 80 ppm up to 126 ppm was detected in six *E. faecalis* strains. Other minor peaks were detected in the chromatograms, none of which was identified as diacetyl.

Biogenic amine production

None of the cheese isolates was able to produce histamine, cadaverine or putrescine in the Joosten & Northolt decarboxylase medium. However, tyramine production was a common characteristic of the tested strains with all the *E. durans*, 97·5 % of the *E. faecalis* and 77·8 % of the *E. faecium* strains showing a positive reaction. There was no *E. gallinarum* strain able to produce tyramine.

Antimicrobial activity

There were several patterns of activity against the target strains (Table 3), with strains active only against one of the indicator strains and others such as FAIR-E 255 and FAIR-E 259 with a broad inhibitory spectrum showing antagonistic activity against *L. innocua*, *C. tyrobutyricum* and *P. freudenreichii* subsp. *shermanii*.

Discussion

The identification of enterococcal species based on simple phenotypic traits often gives ambiguous results. Traditional biochemical tests currently used for taxonomic purposes fail to discriminate several species, particularly those included in the *E. faecium* and *E. gallinarum* group (Devriese *et al.* 1993). In recent years, various

molecular methods have been developed to identify enterococci at both species and strain levels. Among these, RAPD–PCR was successfully used for the identification of clinically relevant species of enterococci (Descheemaeker *et al.* 1997; Quednau *et al.* 1998) and proved to be well-suited for the epidemiological typing of vancomycin-resistant *E. faecium* strains (Barbier *et al.* 1996). Recently, Mannu *et al.* (1999) demonstrated that RAPD–PCR with primers M13 and OPB7 can also be useful to cluster enterococcal strains isolated from Pecorino Sardo cheese; however, the results of this RAPD–PCR analysis were not always in agreement with those obtained with PFGE and plasmid profiles. In the present paper the use of rapid ID 32 STREP galleries allowed the correct identification of 96% of the *E. faecalis* isolates but failed to identify, or gave doubtful identification of, most of the *E. faecium* isolates. The numerical analysis of the RAPD–PCR profiles proved to be a more reliable method to identify enterococci isolated from Italian cheeses.

Most of the strains isolated from Italian cheeses were identified as *E. faecalis* (66·2%); *E. faecium* was also present (21·8%), while *E. durans* was quite uncommon (7·2%). *E. faecalis* has been found to be the predominant enterococcal species in several European artisanal cheeses including Armada cheese (Tornadijo *et al.* 1995), Majoero cheese (Fontecha *et al.* 1990), Monte Veronese cheese (Torriani *et al.* 1998), Caprino cheese (Suzzi *et al.* 2000), Cebreiro cheese (Centeno *et al.* 1996), while higher frequencies of *E. durans* and *E. faecium* over the other enterococcal species are reported in Feta and Kefalotyri cheeses (Litopoulou-Tzanetaki, 1990; Tzanetakis & Litopoulou-Tzanetaki, 1992).

The use of four primers and five amplification conditions allowed the detection of intra-specific differences. However, fifteen strains with identical profiles (coefficient of similarity $r > 0{\cdot}91$) were also found; some of them could be multiple isolates of a same strain since they had been isolated from the same cheese. Three separated clusters were obtained among *E. faecium* isolates, showing a high heterogeneity of this species which needs to be further elucidated on a larger number of strains. However, when the biochemical and physiological data were evaluated, it was not possible to find any phenotypic trait which enabled discrimination between strains belonging to the different *E. faecium* subclusters.

A considerable phenotypic diversity was found among the enterococci isolated from Italian cheeses in terms of ability to grow in milk, proteolytic activity and production of volatile compounds. On the basis of acidifying ability the majority of the strains could be considered as slow acid producers, confirming data previously reported by Arizcun *et al.* (1997) who showed that most of the enterococci isolated from Roncal and Idiazabal cheeses had a low acidifying ability. Proteolytic activity was detected in 24·2% of the strains, all identified as *E. faecalis*. Since proteolytic activity is considered important for cheese ripening, further studies on the proteolytic ability of the strains examined in the present work and on the level of proteolysis obtained by each strain is necessary for a proper selection of strains for cheese-making. As far as it concerns the production of flavour components, the presence of volatile compounds such as acetaldehyde, acetoin and ethanol in milk cultures of the investigated strains is a further indication of the important role that enterococci may play in the development of cheese aroma during ripening.

The detection of several strains with antagonistic activity towards *L. innocua* and other indicator strains agrees with the many data available on the ability of enterococci to produce antimicrobial compounds, mainly bacteriocins, active against various pathogenic and spoilage microorganisms (Giraffa *et al.* 1997; Franz *et al.* 1999). The availability of strains active against *Listeria* spp. is of particular interest

for the dairy industry since these strains may be effective against *L. monocytogenes* contaminations during cheese manufacture and may be used as protective cultures in those cheeses, such as mould-ripened soft cheeses, in which the pH increase during ripening could allow the growth of pathogenic microorganisms.

Although no strain was found to be histamine, cadaverine or putrescine positive, most of the enterococci isolated from cheeses were able to produce tyramine. Formation of tyramine by *E. faecalis* and *E. faecium* is well known (Giraffa *et al.* 1997) and is a matter of discussion and public concern for a possible use of enterococci as starter and/or protective cultures in the cheese industry. However, the method adopted in the present work for the evaluation of biogenic amine production did not give any information on the amount of tyramine produced. Preliminary HPLC evaluation of tyramine production on a limited number of strains (W. H. Holzapfel, personal communication) have indicated some differences in the amount of tyramine produced by each strain, suggesting that a quantitative rather than a qualitative evaluation of tyramine production has to be used as a criterion of selection of enterococci for cheese-making. Furthermore, several parameters such as temperature, pH, concentration of amino acid precursors and presence of amine-destroying enzymes may greatly influence the formation of tyramine in cheese and its concentration during ripening.

Haemolytic activity and vancomycin resistance are potential pathogenic traits that can be displayed by enterococci. Both of them were very rare among the enterococci of dairy origin examined in the present work. Only one out of the 124 strains tested was characterized by a high resistance to vancomycin and teicoplanin and harboured the *van*A gene, suggesting a lower spread of vancomycin resistant enterococci in dairy products. However, since we did not apply any enrichment procedure for the detection of vancomycin resistant enterococci, our results are not comparable with those obtained in other foods, such as meat products, in which high numbers of vancomycin resistant enterococci were found on KAA plates after a step of selective enrichment in the presence of vancomycin (van den Braak *et al.* 1998).

The results obtained in this research showed a considerable genetic and phenotypic diversity among the enterococci isolated from Italian cheeses. Together with the data acquired on potential pathogenic traits, such as haemolytic activity and antibiotic resistance, this information may be used as a base for the selection of safe and useful strains to be applied as starter or protective cultures in the production of cheeses.

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