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ORIGINAL ARTICLE

Dynamics of lactic acid bacteria in "Pecorino di Tramonti"—a ewe's milk cheese—with particular emphasis on enterococci: a preliminary study

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Abstract The autochthonous lactic microflora involved in the production of the artisanal "Pecorino di Tramonti" cheese—a ewe's milk cheese—was evaluated at the date of production and during ripening. Two batches, spicy and mild (not spicy) cheese, of premium quality Pecorino di Tramonti were produced from raw ewe's milk without starter cultures, according to a very traditional technique. Lactic acid bacteria were monitored by counting on selective media. Counts for mesophilic rods and cocci on MRS and LM17 ranged from 6 to 8 Log CFU/g, respectively, during the entire ripening, but different growth trends could be observed between the two kinds of cheese. Enterococcal levels increased during the 1st month and decreased thereafter throughout ripening. A total of 169 cultures was selected randomly and identified by numerical analysis of ribopatterns. Ribotypes could be grouped in five clusters corresponding to different taxons. *Enterococcus faecium* proved to be the dominant species, followed by *Lactococcus lactis* subsp. *lactis*, *L. lactis* subsp. *cremoris*, *Enterococcus faecalis* and *Enterococcus durans*.

Keywords Pecorino cheese · Autochthonous lactic acid bacteria · Enterococci · Ribotyping

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Introduction

In the European Community, the uniqueness of traditional products is usually protected by the attribution of quality marks such as the *Protected Designation of Origin* (PDO) and the *Protected Geographic Indication* (PGI). Apart from some well-known Italian ewe's cheeses, such as Pecorino Romano, Fiore Sardo and Canestrato Pugliese, there is also a large variety of "pecorini" without a "Protected Designation". Most of these dairy products are characterised by distinctive traits and are man-made in marginal and less known areas, so their protection is crucial to preserve the biodiversity of territory and animals. One such product—Pecorino di Tramonti— is a semi-hard cheese produced in picturesque areas of the Amalfi Coast (Salerno Province, Campania Region, Italy). Pecorino di Tramonti is generally manufactured from raw ewe's milk without the addition of primary natural or commercial starter cultures, so its typical characteristics are affected, together with the manufacturing process and the feed provided to animals, mainly by the lactic acid bacteria (LAB) present in the raw milk and by the adventitious microbiota of the dairy environment. Pecorino di Tramonti is sometimes enriched with red pepper to produce a spicy version of the cheese.

The indigenous lactic microbiota of artisanal cheeses, without deliberately added starters, is characterised by high numbers of wild *Lactococcus* and *Enterococcus* sp. (Cogan et al. 1997). As widely recognized, both genera play a key role in acid production, flavour development and the production of bacteriocins (Cogan et al. 1997).

The aim of the present study was to monitor the dynamics of autochthonous LAB during the production and ripening of artisanal "Tramonti" cheese. Genetic variability within lactococci and enterococci populations was explored by numerical analysis of ribopatterns, a powerful tool for taxonomic studies (Rahkila et al. 2011).

Materials and methods

Cheese making and sampling

A small dairy farm located in Tramonti (Province of Salerno), known for its traditional technology and premium quality production, was selected for this study. Two kind of products—Pecorino di Tramonti (ICT) and Pecorino di Tramonti spicy type (ICTS)—were monitored; both kinds of cheese were produced from raw ewe's milk without any commercial starter or natural whey culture, according to traditional practices. Briefly, bulk milk was poured by filtration through fine mesh cloths into a copper tank, which was heated to approximately 32 °C and commercial liquid rennet (0.1 mL/L milk) was added. Dry salt (4 %) was added to the curd and it was cut into pieces of the size of corn kernels using a traditional wooded tool. At this step, in the production of ICTS type, about 100 g crushed red pepper was added. Successively, the curd was collected in perforated plastic baskets and left to dry at 22–28 °C, for 6–8 h, depending on season conditions. The cheese forms were then aspersed with salt and left to drain for a further 48 h. "Pecorino di Tramonti" may be marketed either fresh or as a ripened cheese (30–90 days).

For both type of cheeses (ICT and ICTS), curd immediately after brining (day 0) as well as cheese after 30, 60 and 90 days of ripening were sampled aseptically, transported at 4 °C to the laboratory and submitted to microbiological analyses within a couple of hours. Three consecutive batches, produced between May and June 2012, were analysed.

Microbial and physico-chemical analysis

Samples (25 g), excised aseptically from the core of the product, were homogenized in 225 mL 2 % (w/v) sterile

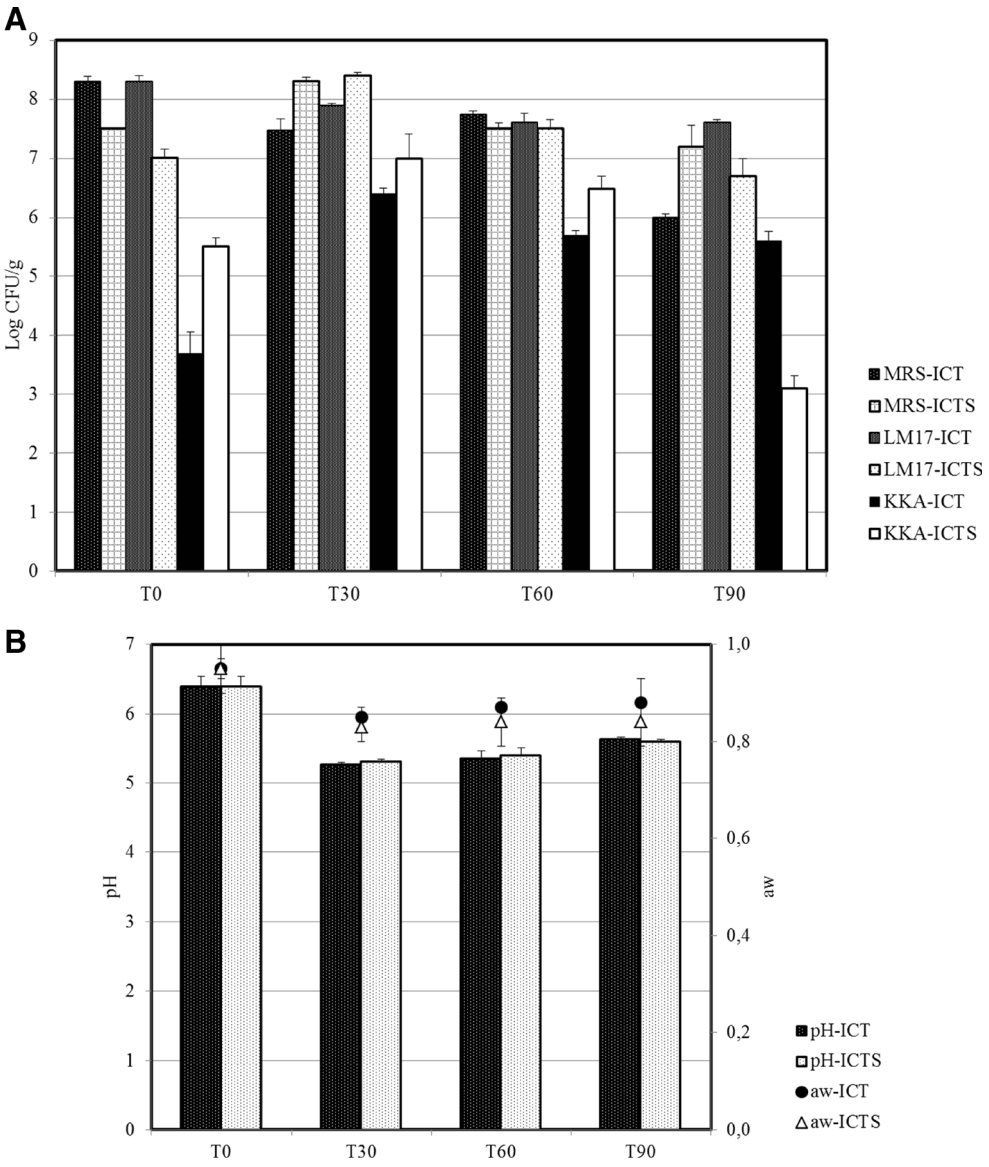

Fig. 1 Evolution of **a** lactic acid bacteria (LAB) and **b** measured chemical parameters during ripening of "Pecorino di Tramonti" cheese: batch ICT (Pecorino di Tramonti) and ICTS (Pecorino di Tramonti spicy type). Values are means of three determinations (± SD) calculated with 95 % confidence

sodium citrate solution at 45 °C for 1 min in a Stomacher Lab-Blender 400 (PBI International, Milan, Italy). Ten-fold serial dilutions in sterile quarter-strength Ringer's solution were plated in triplicate for both microbial enumeration and isolation using the spreading plate method. The following media and incubation conditions were used: M17 agar with 1 % lactose (LM17) incubated at 30 °C for 48 h; MRS agar at 30 °C for 72 h in anaerobiosis, and kanamycin esculin azide agar (KAA) after incubation at 37 °C for 48 h. All media were provided by Oxoid (Basingstoke, UK). Determinations of pH and water activity (a_w) were carried out using a pH meter (MP120; Mettler-Toledo Spa, Schwerzenbach, Switzerland) and a Hygrolab Ro-tronic (PBI International), respectively.

Isolation and presumptive identification of LAB

A total of 169 colonies was isolated from LM17 and KAA agar plates seeded with the highest sample dilutions, to analyse cocci-shaped LAB. In detail, between 1 and 20 colonies (corresponding to the square root of the total numbers of colonies) were picked randomly and purified by streaking repeatedly onto LM17 agar plates. All isolates were characterised by Gram staining, catalase production (bioMérieux Marcy l'Etoile, France), spore formation and cell morphology by phase contrast microscopy.

Isolation of chromosomal DNA and restriction enzyme digestion

Cultures were grown in appropriate broth up to exponential phase. Cells were harvested by centrifuging for 10 min at 15,000*g* in a Biofuge A bench centrifuge (Heraeus, Hanau, Germany) to provide a pellet of approximately 15 mg (wet weight). Chromosomal DNA was isolated by the guanidium thiocyanate method of Björkroth and Korkeala (1996). Restriction digestions using endonucleases *Hin*dIII and *Eco*RI were performed according to instructions provided by the manufacturer (New England BioLabs, https://www.neb.com).

Southern transfer and hybridization with DIG-oligonucleotide probes

DNA restriction patterns were transferred from gel to MSI Magnagraph membranes (MSI, Westboro, USA) by a VacuGene XL blotting system (Pharmacia, Uppsala, Sweden). DNA was fixed using UV irradiation in optimal crosslink mode in a Spectrolinker XL 1000 (Spectronics Corporation, New York, NY, USA). Membranes were hybridized at 53 °C overnight in a Techne Hybridizer (Techne, Cambridge, UK) using a rDNA 3′ 5′-labelled oligo probe mixture called OligoMix5 as indicated by Regnault et al. (1997). A DIG DNA Labelling and Detection Kit (Boehringer Mannheim, Germany) was used for hybridization, washes and development of the digoxigenin label.

LAB database and numerical pattern analysis

The ribopatterns were compared to patterns of a previously established LAB database (Department of Food and Environmental Hygiene, University of Helsinki, Finland). This database contains the ribopatterns of approximately 7,000 relevant food-associated LAB (Koort et al. 2004, 2005, 2006) and the reliability of clusters for distinguishing between different species has been evaluated in several polyphasic taxonomy studies (Koort et al. 2004, 2005, 2006). For the numerical analysis, the ribopatterns were scanned using a Hewlett-Packard (Boise, ID) ScanJet 4c/T scanner and normalised based on the mobility of standards. A similarity matrix was created using the BioNumerics (version 4.61) software package (Applied Maths, Sint-Marten-Latem, Belgium). Similarity between all pairs was expressed by Dice coefficient correlation, and UPGMA (unweighted-pair group method) clustering was used for construction of the dendrogram.

Table 1 Bacterial species isolated during manufacture and ripening of "Pecorino di Tramonti" cheese. *KAA* Kanamycin esculin azide agar, *LM17* M17 agar with 1 % lactose

Culture identification	Sample[a]	Days of ripening				Isolation medium
		0	30	60	90	
Enterococcus faecium	ICT	12[b]	4	11	4	KAA
Enterococcus faecalis		1	1	4		
Enterococcus durans			1			
Lactococcus lactis spp. *lactis*				1		
E. faecium	ICT	2	1	16	21	LM17
E. faecalis					2	
E. durans					2	
L. lactis spp. *lactis*			18			
E. faecium	ICTS	1	7	2	8	KAA
E. faecalis			3		1	
E. durans			1			
L. lactis spp. *cremoris*				1		
E. faecium	ICTS	5	6	3	11	LM17
E. durans		1			1	
L. lactis spp. *cremoris*		5	11			

[a] *ICT* Pecorino di Tramonti, *ICTS* Pecorino di Tramonti spicy type

[b] Number of isolates

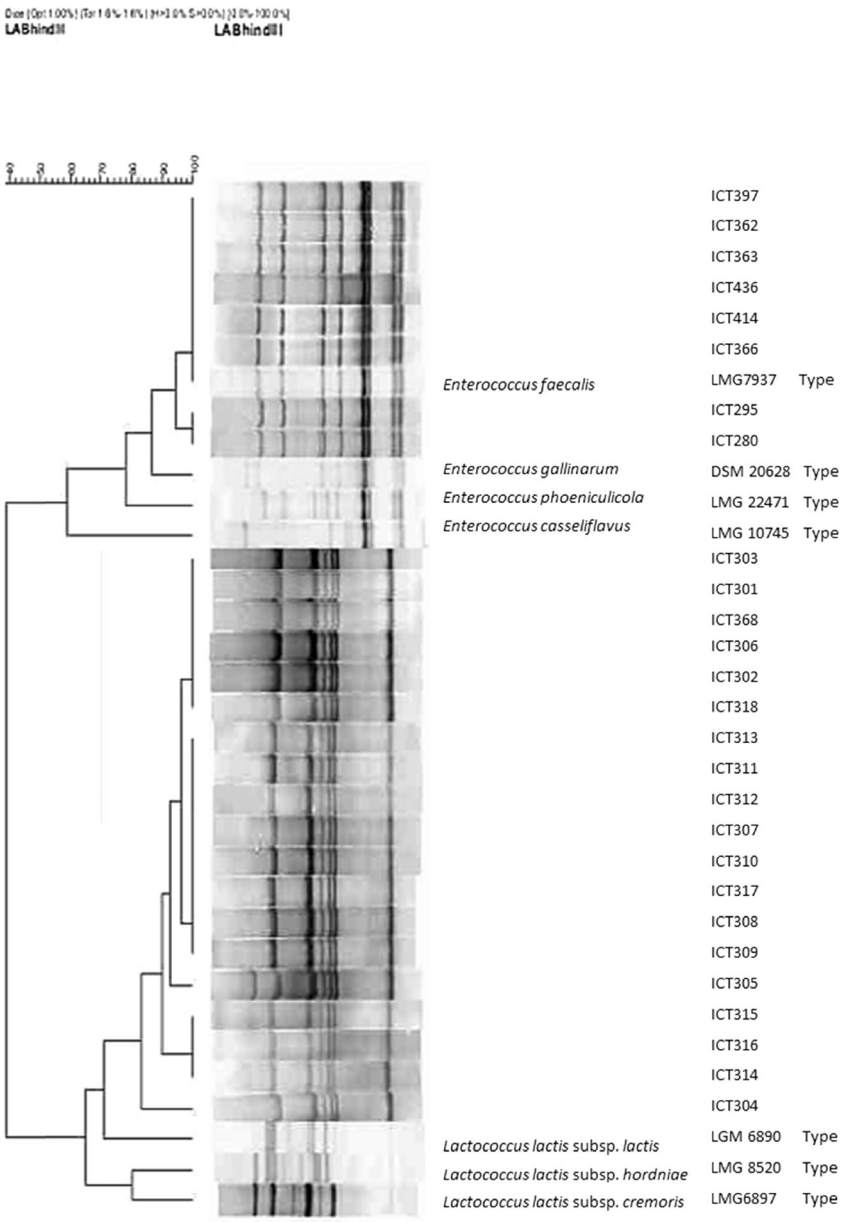

Fig. 2 Numerical analysis of rRNA *Hind*III restriction patterns (ribotypes) of *Enterococcus faecalis* and *Lactococcus lactis* subsp. *lactis* in batch ICT. Numerical analyses of the patterns are presented as a dendrogram

Results and discussion

Microbial and physico-chemical analysis

Evolution of LAB counts throughout ripening is presented in Fig. 1. In both kinds of cheeses, counts of lactobacilli on MRS and lactococci on LM17 showed loads ranging from 6 to 8 Log CFU/g, but different behaviour was highlighted: in ICT samples, the highest population level on both media (8.3 Log CFU/g) was recorded on the day of production and then loads decreased steadily to 6.0±0.2 and 7.6±0.2 Log CFU/g for MRS and LM17, respectively (Fig. 1a); on the other hand, in ICTS batches, lactobacilli and lactococci reached maximum levels only after 1 month of ripening (8.3±0.1 and 8.4±0.4 Log CFU/g), likely due to the antimicrobial effect of the spices added to the curd (Tarakci and Temiz 2009). The highest population level of enterococci (6.4±0.1 and 7.0±0.4 Log CFU/g for ICT and ICTS, respectively) was reached after 1 month of ripening, and loads subsequently decreased in both cases. In detail, the most significant drop was recorded for spicy cheese samples: counts dropped to 3.1±0.2 Log CFU/g in 2 months (Fig. 1a). Enterococci counts were higher than those reported for "Pecorino di Filiano" (Bonomo and Salzano 2012) and "Pecorino del Poro" (Caridi et al. 2003), but comparable to values reported for "Pecorino Crotonese" (Randazzo et al. 2010). The presence of high numbers of enterococci in cheeses

made from raw ewe's milk is probably induced by the resistance of members of this genus to high temperatures and high concentrations of salts or acids (Caridi et al. 2003). pH and a_w values showed overlapping trends for ICT and ICTS samples (Fig. 1b). On the day of production, the pH was around 6.40 but decreased to about 5.6 at the end of ripening in both cases (Fig. 1b). The a_w decreased steadily from an initial value of 0.95, reaching, at the end of ripening 0.88±0.05 and 0.84±0.04, for ICT and ICTS batches, respectively.

Identification of LAB cultures

In total, 169 cocci shaped LAB strains, 102 and 67 from ICT and ICTS samples, respectively, were identified at genus, species and sub species level by using the LAB database of ribopatterns. Five clusters corresponding to five LAB species/subspecies were obtained (Table 1). Out of 169 strains, 133 were identified as enterococci: 115 *E. faecium*, 12 *E. faecalis* and 6 *E. durans*. The remaining 36 strains could be assigned to the species *L. lactis*: in detail, 19 proved to be *L. lactis* subsp. *lactis* and 17 were *L. lactis* subsp. *cremoris*. *Hind*III pattern analysis resulted in identification of *L. lactis* subsp. *lactis*, *L. lactis* subsp. *cremoris* and *E. faecalis* clusters (Figs. 2, and 3), while identification of *E. faecium* and *E. durans* clusters required both *Hind*III- and *Eco*RI-deduced patterns in a comparative analysis (Fig. 4). Enterococci were detected in all samples, whereas an uncommon behaviour was recorded within lactococci: *L. lactis* subsp. *lactis* was recovered in ICT batches (Fig. 2), while *L. lactis* subsp. *cremoris* was retrieved exclusively in ICTS batches (Fig. 3). The occurrence of lactococci in the earliest phases of maturation of "Tramonti" cheese has been reported for several other ewes' cheeses (Randazzo et al. 2010; Ricciardi et al. 2014). As expected, all lactococci were isolated from LM17 agar plates, and mainly after 1 month of ripening. On the other hand, this medium also allowed the growth of

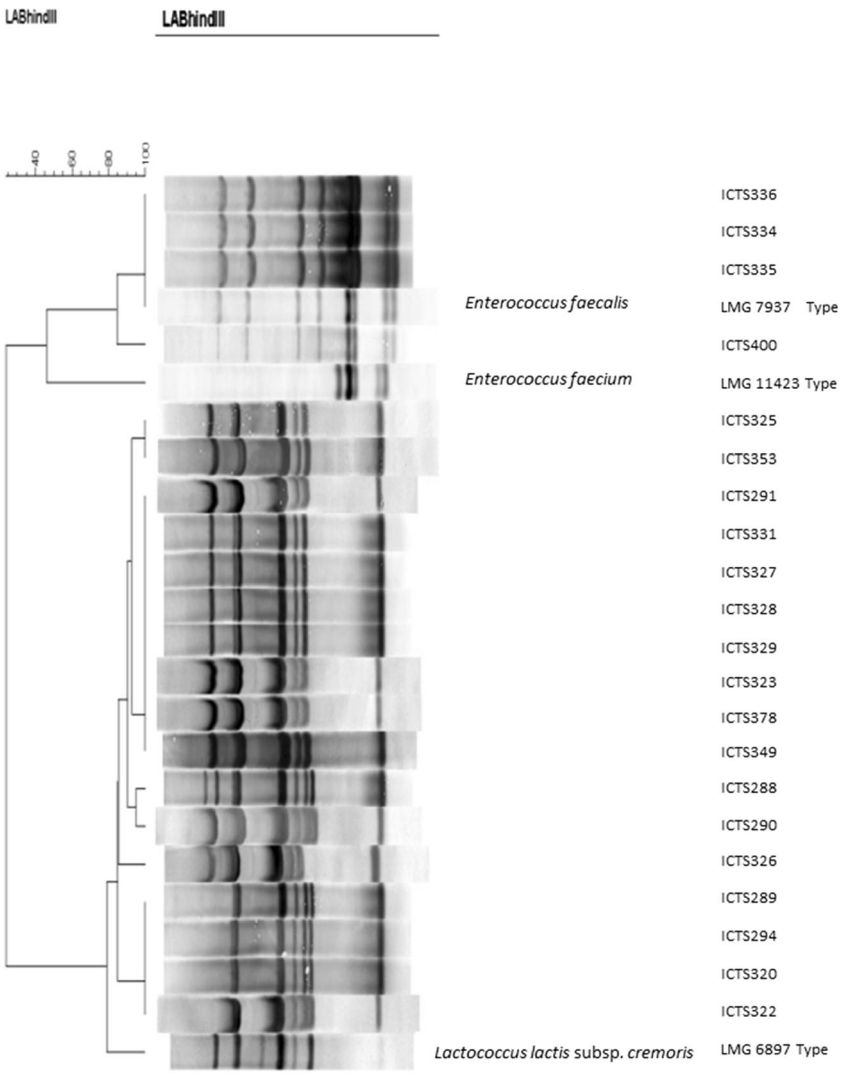

Fig. 3 Numerical analysis of rRNA *Hind*III restriction patterns (ribotypes) of *E. faecalis* and *L. lactis* subsp. *cremoris* in batch ICTS. Numerical analyses of the patterns are presented as dendrogram

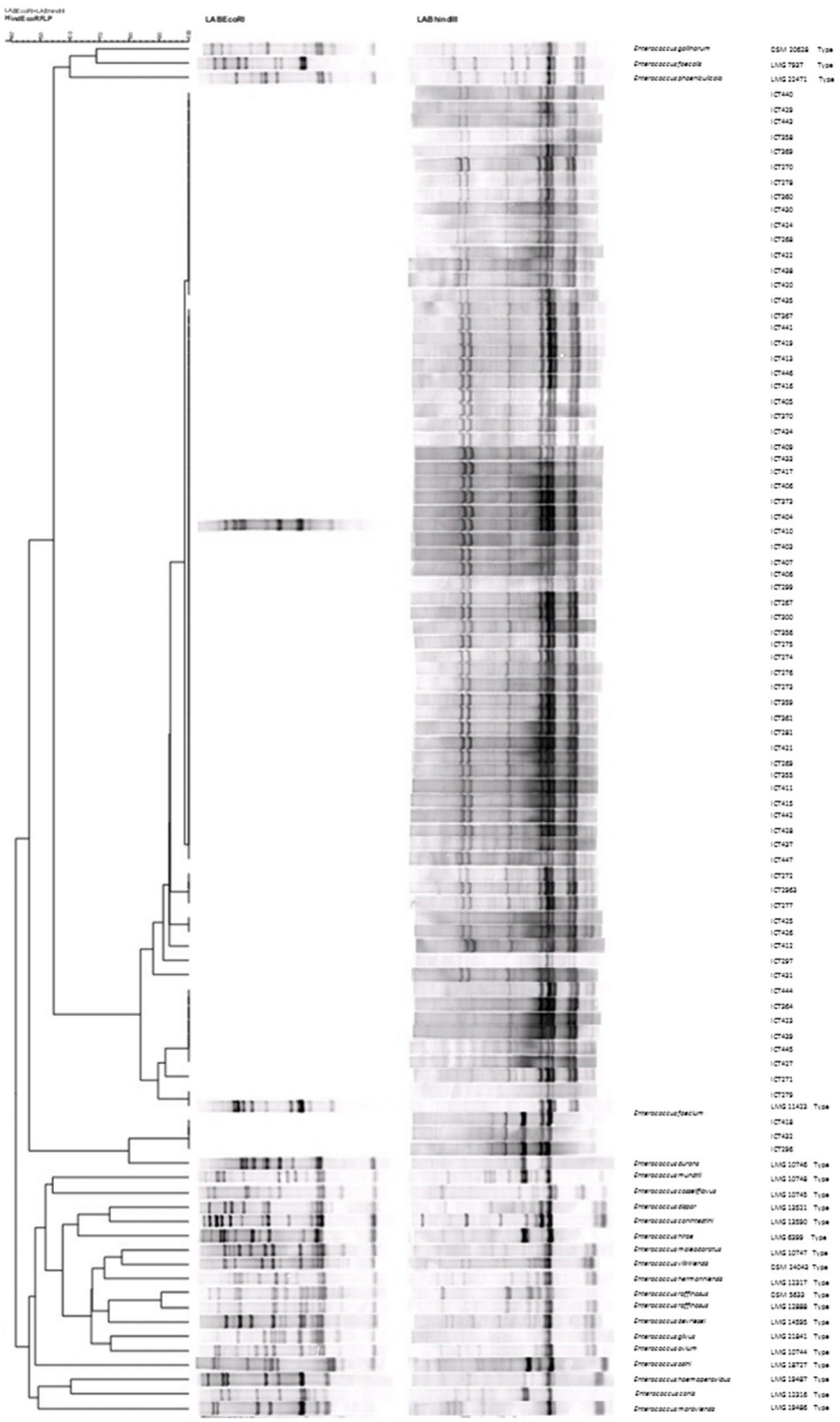

Fig. 4 Comparative numerical analysis of rRNA *Hind*III and *Eco*RI restriction patterns (ribotypes) of *E. faecium* and *E. durans* strains in batch ICT. Numerical analyses of the patterns are presented as dendrogram

enterococci, thus confirming its poor selectivity (Caridi et al. 2003). *Enterococcus* sp. are often isolated from artisanal ewe's cheeses due to their ability to contaminate raw milk directly as faecal contaminants or indirectly from the milking and cheese-making environment (Aquilanti et al. 2006). The presence and persistence of these microorganisms during ripening can be ascribed to their remarkable resistance to the high salt content and low pH of fully ripened cheese (Aquilanti et al. 2006) as well as to the storage temperature of the milk before manufacture and the slow acid production during cheese-making (Caridi et al. 2003). The dominance of enterococci in "Tramonti cheese" may underline its positive role in curd acidification and in the development of the typical cheese aroma and flavour (Moreno et al. 2006). Nevertheless, despite the inclusion of *E. faecium* in the list of the LAB starters by the

International Dairy Federation (Giraffa 2002), the use of these microorganisms as commercial starter or adjuncts in the dairy industry is still debated due to their role as potential pathogens and/or as reservoirs of transferable antibiotic-resistance genes (Moreno et al. 2006). *E. faecium* was by far the most detected species, which did not match the enterococcal species distribution reported for "Canestrato Pugliese" (Aquilanti et al. 2006), "Pecorino Crotonese" (Randazzo et al. 2010) and "Pecorino Abruzzese" (Serio et al. 2007).

The results of this study describe the function and diversity of homofermentative cocci, such as lactococci and enterococci, in "Pecorino di Tramonti" cheese. Adventitious LAB counts were significant and comparable to those reported for other artisanal raw ewe's milk cheeses. Enterococci dominated the indigenous lactic microbiota, with *E. faecium* as prevailing species from the 1st ripening month onwards. The identified LAB strains, after further investigation of their technological features, could be used as autochthonous starter strains to standardise, improve and reinforce the artisanal manufacturing of this distinctive pecorino cheese.

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Conflict of interest The authors declare that they have no competing interests.

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