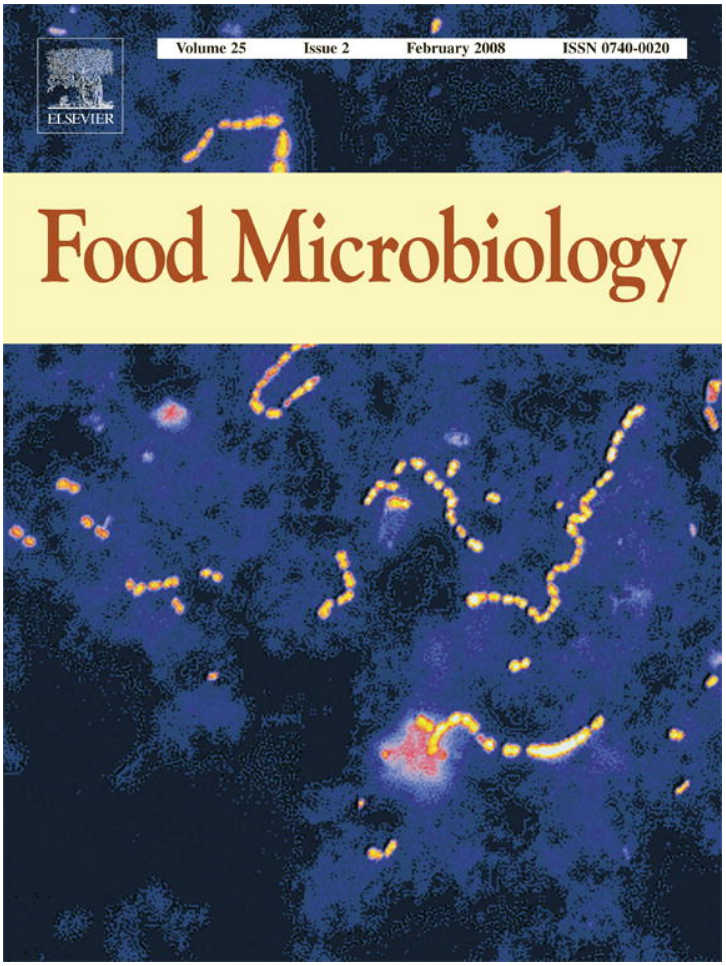

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Microbiological characterization of artisanal Raschera PDO cheese: Analysis of its indigenous lactic acid bacteria

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Abstract

The aim of this research was to study the bacterial populations involved in the production of artisanal Raschera PDO cheese (Italian Maritime Alps, northwest Italy) in order to collect preliminary knowledge on indigenous lactic acid bacteria (LAB). A total of 21 samples of Raschera PDO cheese, collected from six dairy farms located in the production area, were submitted to microbiological analysis. LAB were randomly isolated from M17 agar, MRS agar and KAA plates and identified by combining PCR 16S–23S rRNA gene spacer analysis, species-specific primers and 16S rRNA gene sequencing. Biodiversity of *Lactococcus lactis* subsp. *lactis* isolates was investigated by RAPD-PCR.

LAB microflora showed the highest count values among all microbial groups targeted. They reached counts of 10^9 colony forming unit (cfu)/g in cheese samples after 3 days of salting and 15 days of ripening. Yeast population also showed considerable count values, while enterococci and coagulase-negative cocci (CNC) did not overcome 10^7 cfu/g. *L. lactis* subsp. *lactis* was the species most frequently isolated from Raschera PDO samples at all different production stages while in aged cheeses *Lactobacillus paracasei* was frequently isolated. RAPD-PCR highlighted that isolates of *L. lactis* subsp. *lactis* isolated from Raschera PDO were highly homogeneous.

Keywords: Lactic acid bacteria; Raschera PDO cheese; Microbiological characterization; RAPD-PCR; 16S–23S rRNA gene spacer analysis

1. Introduction

Raschera PDO is a semi-hard and pressed cheese produced in Piedmont, in Monregalese Valley (Italian Maritime Alps, northwest Italy). Raschera belongs to those niche products that are strongly connected to Italian food tradition and have been recently re-discovered and recognized from the European Community with the attribution of the ''Protected Denomination Origin'' status (European Regulation 510/06). In the last years it has been estimated, on average, an annual production of 2500 quintals, as reported by the Farmers' Cooperative of Raschera PDO.

In the traditional Raschera PDO cheesemaking, raw cow milk coming from two milkings is warmed to 27–30 °C and left resting for 20–30 min after the addition of liquid rennet. Clotting time is established visually by the cheese maker. The curd is cut into particles, then separated from the serum and collected in a hemp cloth which are left for approximately 10 min of drainage. Finally, the curd wrapped in the cloth is put into cylindrical forms and pressed to allow remaining serum to drain away for at least 12 h. At the end, the product is ready to be dry salted and ripened in natural cells dug underground (Official Gazette, No. 92, April 2004). Here the constant temperature and humidity create the optimal conditions for the 1–6 months of ripening and consequently for the formation of the reddish rind characteristic of this cheese.

In the last years, industrial dairies have been started producing Raschera PDO cheese by using mesophilic lactic acid bacteria (LAB) commercial starter cultures. Cheese made from raw milk added of starters results in a more uniform and standardized product of better sanitary quality; however, the organoleptic characteristics of the cheese bears little relation to those of its traditional

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counterpart (Wouters et al., 2002; Coppola et al., 2006); in fact, the addition of commercial starters causes the drop of load of the indigenous microflora responsible for the development of typical cheese flavours (Steele and Unlu, 1992; Wouters et al., 2002). The low diversity of starter cultures used in the industrial dairy fermentation is leading to an increasing demand from cheese makers of new LAB strains to be used as indigenous starters. The development of authochtonous cultures specific for Raschera PDO fermentation may ensure protection of sensorial properties and at the same time guarantees an uniform and safer product (De Reu et al., 2002, Fortina et al., 2003; La Rodriguez et al., 2004; Samelis and Kakouri, 2007).

The aim of this research was to study the indigenous bacterial population involved in the production of artisanal Raschera PDO cheese, to obtain preliminary knowledge on LAB species which carry out the traditional fermentation of this cheese. This study represents a first step towards the understanding of microbial population involved in ripening processes of Raschera PDO. Next steps will be the development of selected authochtonous cultures to be used as starters in both industrial and farm processes.

2. Materials and methods

2.1. Dairy samples

A total of 21 samples were obtained during the summer alpine pasture from six representative dairy farms not using starter cultures and located in the production area of Raschera PDO. Nine samples of cheese were taken 3 days after salting that led to an initial microbial selection. Cheese samples at different ripening stages were also collected and precisely six samples at 15 days ripening, three samples at 30 days ripening and three samples at 70 days ripening. All samples were transported to the laboratory under refrigerated conditions not later than 3 h from the collection and subjected to microbiological analysis.

2.2. Microbiological analysis

For microbiologial analysis, 10 g of cheese samples were emulsified with sterile quarter strength Ringer solution (Oxoid, Basingstoke, UK) in a Stomacher machine. Decimal dilutions were prepared in Ringer solution and plated onto different selective media for viable counts. The following analyses were carried out: mesophilic aerobic bacteria on plate count agar (Oxoid) at 30 °C for 48 h, mesophilic and thermophilic cocci on M17 agar (Fluka, Buchs SG, Switzerland), incubated aerobically, respectively, at 30 and 37 °C for 48 h, mesophilic and thermophilic lactobacilli on MRS agar (Oxoid), incubated anaerobically, respectively, at 30 and 37 °C for 48 h, enterococci on kanamycin aesculin azide agar (KAA) (Fluka) at 37 °C for 48 h, coagulase-negative cocci (CNC) on mannitol salt agar (Oxoid) at 30 °C for 48 h, coliforms on violet red bile lactose agar (Fluka) at 37 °C for 24 h, yeast and moulds on malt agar (Oxoid) supplemented with tetracycline (1 μg/ml, Fluka) at 25 °C for 96 h. After counting, from M17, MRS and KAA agars, a total of 15 randomly selected colonies of LAB for each sample were isolated, in order to obtain a representative LAB population of Raschera PDO. They were then purified by two subsequent subcultures on M17 agar and MRS agar and, for long-term maintenance, finally stored at −20 °C in M17 and MRS broth containing 30% glycerol, before being subjected to molecular analysis.

2.3. DNA extraction from pure cultures

Genomic DNA from isolates was extracted from 100 μl of an overnight culture diluted with 300 μl TE 1X buffer (10 mM Tris–HCl, 1 mM Na_2EDTA, pH 8.0) as described by Mora et al., (2000).

2.4. PCR identification of LAB isolates

Identification of LAB isolates was carried out by combining PCR 16S–23S rRNA gene spacer analysis (RSA), species-specific primers and 16S rRNA gene sequencing. The RSA was carried out with primers G1 and L1 (Table 1). This analysis allowed to group the isolates on the basis of polymorphism of 16S–23S rRNA gene spacer region, and to obtain an indication of the hypothetical genus they could belong to (Coppola et al., 2001, Fortina et al., 2003). According to RSA results, species-specific PCRs and 16S rRNA gene sequencing were planned in order to determine the taxonomic positions of the isolates at species level. The primers used and their corresponding references are reported in Table 1. Isolates for which it was not possible to establish a well-defined RSA profile were submitted to partial 16S rRNA gene amplification with primers p8FPL and p806R (Table 1). The amplified fragments were sent for the sequencing to MWG Biotech (Ebersberg, Germany) and the sequences obtained were aligned with those in GenBank with Blast program (Altschul et al., 1997) to determine the closest known relatives of isolates.

RSA reactions were performed in a final volume of 25 μl containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphates, 0.5 U of Taq polymerase (Eppendorf, Milan, Italy), 0.5 μM concentrations of each primer. One microliter of template DNA was added to the mixture. RSA thermal cycling was as follows: an initial denaturation of 95 °C for 5 min, 40 cycle of 2 min at 95 °C, 1 min at 55 °C, 2 min at 72 °C, and 10 min of final extension. PCR products were analysed by electrophoresis in 1.5% Tris–acetate–EDTA agarose gels and a 1 kb ladder (MBI Fermentas, St. Leon-Rot, Germany) was used as size marker.

2.5. RAPD-PCR

DNA extracted from *Lactococcus lactis* subsp. *lactis* isolates was used in RAPD-PCR analysis. Amplification

Table 1
Nucleotidic sequence, target gene and reference of primers used for LAB isolates identification

Primers (5′–3′)	Target gene	Reference
G1: GAAGTCGTAACAAGG	Bacterial 16S–23S rRNA gene spacer region	Jensen et al. (1993)
L1: CAAGGCATCCACCGT p8FPL: AGAGTTTGATCCTGGCTCAG	Bacterial 16S rRNA region	Teymoortash et al. (2002)
p806R: CTACGGCTACCTTGTTACGA EM1A: TTGAGGCAGACCAGATTGACG	Nucleotide sequence conserved in *E. faecium*	Cheng et al. (1997)
EM1B: TATGACAGCGCTCCGATTCC E1: ATCAAGTACAGTTAGTCTT	*Ddl* gene in *E. faecalis*	Dutka-Malen et al. (1995)
E2: ACGATTCAAAGCTAACTG His1: CTTCGTTATGATTTTACA	Histidine biosynthesis operon in *L. lactis*	Corroler et al. (1999)
His2: CAATATCAACAATTCCAT pLG-1: CATAACAATGAGAATCGC	16S rRNA gene in *L. garvieae*	Zlotkin et al. (1998)
pLG-2: GCACCCTCGCGGGTTG para: CATAACAATGAGAATCGC	16S rRNA gene in *L. paracasei*	Ward and Timmins (1999)
Y2: GCACCCTCGCGGGTTG 16: CATAACAATGAGAATCGC Lpl: GCACCCTCGCGGGTTG	16S–23S rRNA gene spacer region in *L. plantarum*	Berthier and Ehrlich (1998)

reactions were carried out using primer D8635 (5′-GAGC-GGCCAAAGGGAGCAGAC-3′) as previously described by Lombardi et al. (2002). RAPD-PCR products were separated by electrophoresis on 1.5% (w/v) agarose gels in 1X TBE buffer at 120 V for 2 h. Pictures of the gels were digitally captured by UVIpro Platinum 1.1 Gel Software (Eppendorf) and BioNumerics Software (Applied-Maths, Sint-Martens-Latem, Belgium) was used for pattern analysis. Calculation of similarities of band profiles was based on the Pearson product moment correlation coefficient. Dendrograms were obtained by means of the unweighted pair group method by using arithmetic average (UPGMA) clustering algorithm (Vauterin and Vauterin, 1992). RAPDs were performed at least twice for each strain.

3. Results

3.1. Microbial counts

The mean microbial counts of the 21 samples of Raschera PDO cheese at different ripening stages are summarized in Figs. 1 and 2. The minimum and maximum count values are also reported for each microbial group detected.

The mesophilic aerobic bacteria count ranged from 10^8 to 10^9 cfu/g (Fig. 1) and reached the maximum value of 4.2×10^9 cfu/g in a sample collected 3 days after salting. LAB showed values of 10^8– 10^9 cfu/g in samples taken after 3 days of salting and 15 days of ripening with the only exception of the lowest load 4.0×10^6 cfu/g detected on MRS agar. LAB kept values ranging from 10^7 to 10^8 cfu/g at 30 and at 70 days of maturation (Fig. 2). Mesophilic and thermophilic LAB microflora counts, as well as presumptive

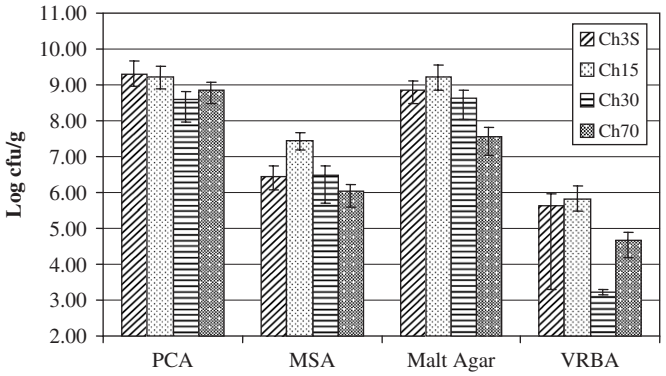

Fig. 1. Log microbial counts (mean ± maximum and minimum values) of non-lactic microflora during ripening of Raschera PDO cheese. Ch3S: 3 days after salting cheeses; Ch15: 15 days ripened cheeses; Ch30: 30 days ripened cheeses; Ch70: 70 days ripened cheeses.

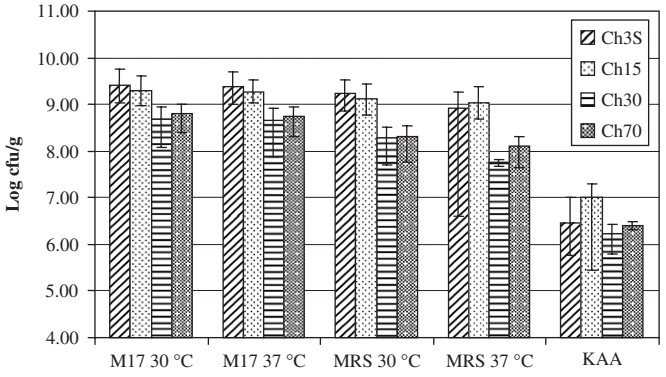

Fig. 2. Log microbial counts (mean ± maximum and minimum values) of LAB during ripening of Raschera PDO cheese. Ch3S: 3 days after salting cheeses; Ch15: 15 days ripened cheeses; Ch30: 30 days ripened cheeses; Ch70: 70 days ripened cheeses.

lactococci and lactobacilli counts revealed on M17 and MRS agar plates, did not highlight strong differences.

Enterococci were present with lower counts (10^6–10^7 cfu/g) compared to lactococci and lactobacilli (Fig. 2). The CNC were generally present with values of about 10^6 cfu/g even if they reached counts of 10^7 cfu/g in 15 days ripened samples (Fig. 1). Yeasts also reached the highest levels (10^9 cfu/g) in cheese collected after 15 days of ripening, then for decrease to 10^7 cfu/g in 70 days ripened samples (Fig. 1). Coliforms were present at 10^5 cfu/g in samples taken after 3 days of salting and 15 days of ripening. Their loads lowered in aged cheeses however the values were higher than 10^3 cfu/g (Fig. 1).

3.2. Molecular identification of LAB isolates

A total of 283 presumptive LAB isolates were randomly selected from agar plates and subjected to molecular identification. A first clustering identification step was reached by RSA obtaining seven different profiles (Fig. 3). Cluster I was composed of 198 isolates and its profile was characterized by a unique band migrating approximately at 390 bp (Fig. 3, lane 2). In cluster II, seven isolates were characterized by a unique band migrating approximately at 450 bp (Fig. 3, lane 3). Cluster III was composed of 10 isolates whose profiles were characterized by a unique band migrating approximately at 380 bp (Fig. 3, lane 4).

In cluster IV, 34 isolates were grouped, showing by two bands migrating at 400 and 500 bp (Fig. 3, lane 5). For the other 17 isolates, two amplification bands at approximately 300 and 400 bp were obtained (cluster V) (Fig. 3, lane 6). Clusters VI and VII, including seven and three isolates, respectively, exhibited a three or four bands profile (Fig. 3, lanes 7 and 8).

Species-specific primers and 16S rRNA gene sequencing were necessary to finally identify the isolates. Within cluster I, the isolates were discriminated between the two major subspecies of *L. lactis*, subsp. *lactis* and subsp. *cremoris*, on the basis of the different sizes of partial histidine biosynthesis operon amplified (Corroler et al., 1999). Clusters II and III grouped isolates belonging, respectively, to the species *Lactococcus garvieae* and *Streptococcus infantarius* subsp. *infantarius*. Clusters IV and V included enterococcal isolates belonging, respectively, to the species *Enterococcus faecium* and *Enterococcus faecalis*. The isolates within group VI were identified as *Lactobacillus paracasei*, while isolates within cluster VII were tested positive to amplification with *Lactobacillus plantarum* specific primers. The isolates belonging to cluster III showed an unknown RSA profile; thus, they were submitted to 16S rRNA gene sequencing and resulted to belong to *S. infantarius* subsp. *infantarius* (Table 2). For seven isolates it was not possible to establish a well-defined RSA profiles, thus they were also submitted to 16S rRNA gene sequencing. Three isolates resulted to belong to the species *Enterococcus casseliflavus*, one to *Leuconostoc pseudomesenteroides* and one to *Vagococcus carniphilus*. The remaining two isolates were classified as species belonging to *Staphylococcus* genus (Table 2).

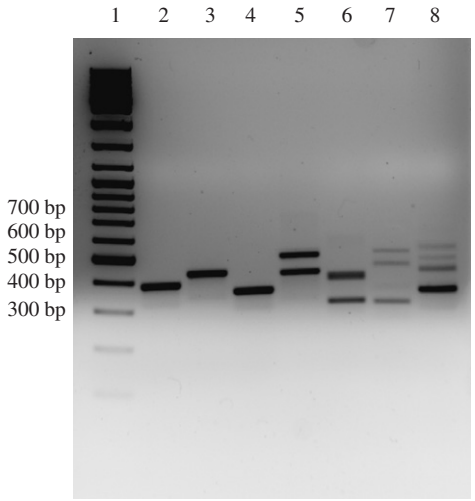

Fig. 3. RSA profiles obtained from the LAB isolated from Raschera PDO cheese samples—lane 1: 1 kb DNA ladder (MBI); lanes 2–8: amplification profiles obtained by RSA analysis.

Lactococci were dominant in all cheese samples at the different ripening stages; in particular *L. lactis* subsp. *lactis* was present with frequencies ranging from 61% to 86% among LAB isolates; *L. garvieae* and *L. lactis* subsp. *cremoris* were isolated with percentages not higher than 9% and 3%, respectively (Fig. 4). Non-starter LAB (NS-LAB) isolates were obtained from aged cheeses. *Lb. paracasei* showed a considerable presence (23%) in cheese samples collected after 30 days ripening and *Lb. plantarum* was isolated from both 30 and 70 days ripened cheeses with 3% and 6% of frequency, respectively (Fig. 4). Enterococci were present with low percentages at the different ripening stages with the species *E. faecium*, *E. faecalis* and *E. casseliflavus*. *V. carniphilus* and *L. pseudomesenteroides* were occasionally isolated while *S. infantarius* subsp. *infantarius* was found in cheeses taken 3 days after salting and at 5, 15, 30 days ripening with frequencies of 8%, 7% and 3%, respectively (Fig. 4).

In Fig. 5 the frequency of isolation of LAB species in correlation to each selective LAB media is reported. M17 and MRS agar showed low selectivity towards lactococcal isolates obtained from both media with comparable percentages while lactobacilli grew preferably on MRS agar. KAA medium was highly selective for enterococci. The incubation temperature used in the study did not allow to obtain a marked growth differentiation among LAB species isolated belonging to mesophilic microflora.

3.3. RAPD-PCR

RAPD-PCR was carried out to explore the genetic diversity of 183 *L. lactis* subsp. *lactis* isolates from Raschera PDO samples. Each strain showed the same RAPD profile in the RAPD analysis repetitions carried

Table 2
Sequence information for isolates with unclear or unknown RSA profile

Strain	Closest sequence relative	% identity	GenBank accession no.
I	*Streptococcus infantarius* subsp. *infantarius*	99	DQ232530.1
II	*Streptococcus infantarius* subsp. *infantarius*	99	DQ232529.1
III	*Streptococcus infantarius* subsp. *infantarius*	99	AF177729.1
IV	*Enterococcus casseliflavus*	100	EU075043.1
V	*Enterococcus casseliflavus*	100	DQ223887.1
VI	*Enterococcus casseliflavus*	100	DQ839454.1
VII	*Vagococcus carniphilus*	99	AY179329.1
VIII	*Leuconostoc pseudomesenteroides*	100	AY929289.1
IX	*Staphylococcus* sp.	99	AY578092.1
X	*Staphylococcus* sp.	99	DQ356500

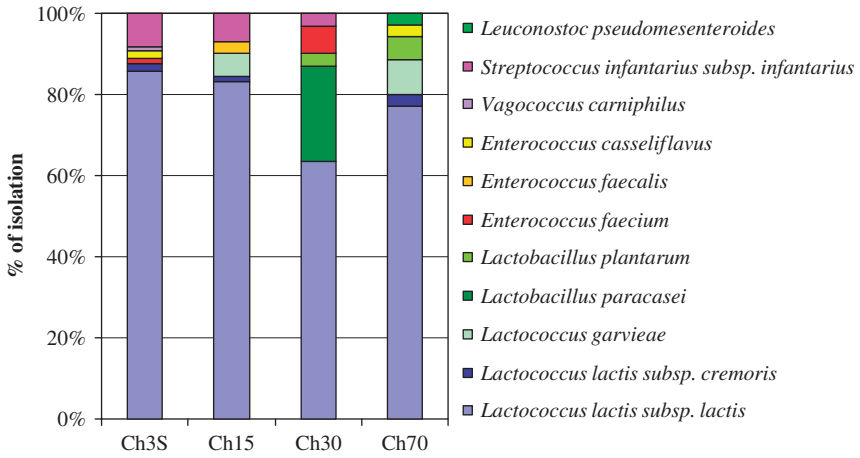

Fig. 4. Frequency of isolation of LAB species from cheese samples of Raschera PDO at different ripening stages. Ch3S: 3 days after salting cheeses; Ch15: 15 days ripening cheeses; Ch30: 30 days ripening cheeses; Ch70: 70 days ripening cheeses.

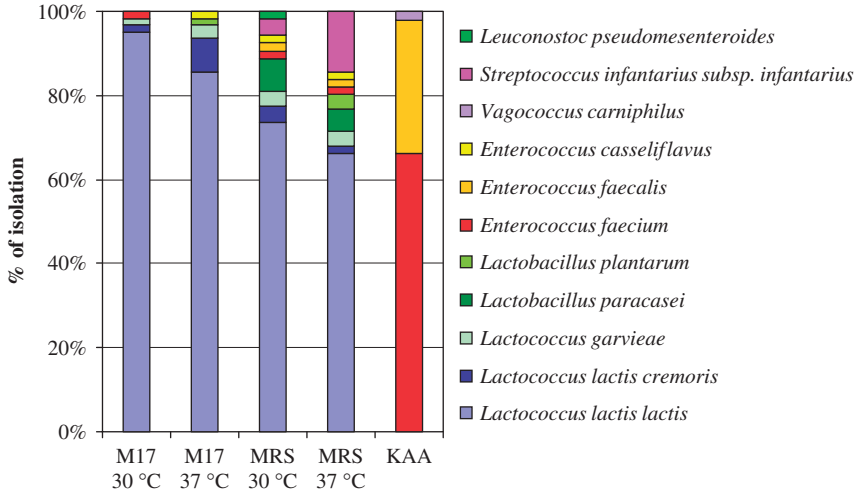

Fig. 5. Prevalence of LAB species among isolates randomly selected on KAA, and M17 agar and MRS agar plates incubated at both 30 and 37 °C.

out. Elaboration of RAPD profiles obtained with primer D8635 resulted in the dendrogram shown in Fig. 6. A general low biodiversity was revealed among *L. lactis* subsp. *lactis* strains. At similarity level of 80% between two main clusters, I and II (Fig. 6), was detected including, respectively, 108 and 63 lactococcal isolates equally distributed among cheese samples at different ripening stages, coming from the diverse dairy farms. Twelve isolates did not share any similarity with the others studied, and clustered outside the two main groups.

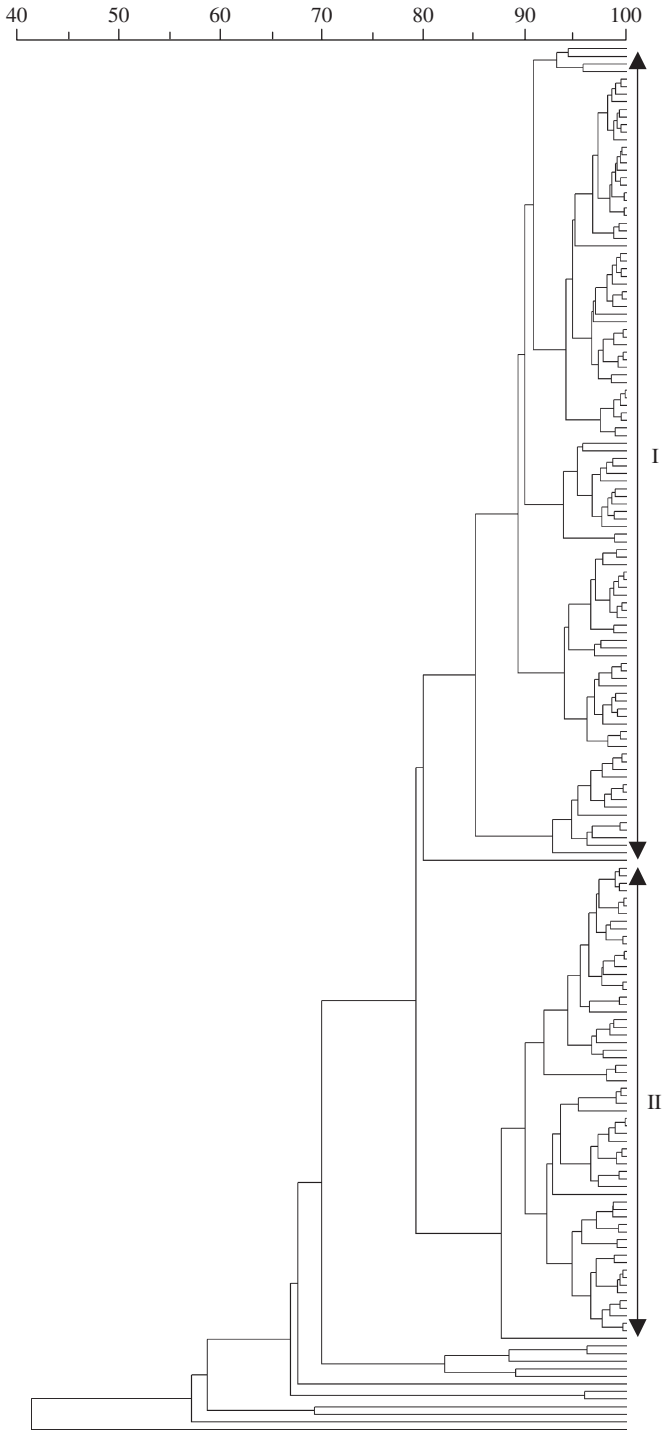

Fig. 6. RAPD-PCR cluster analysis of profiles obtained with primer D8635 from *Lactococcus lactis* subsp. *lactis* strains isolated from Raschera POD samples.

4. Discussion

Typical and traditional foods represent an important patrimony for a region and a great opportunity for the development of rural areas. This is the case of Raschera PDO cheese which is produced in a small area on Italian Maritime Alps. The thorough knowledge of a dairy product represents the first step towards the improvement of its quality and, as a consequence, the expansion of its market. In this research we better define the main microflora, which plays a significant role in the ripening process of Raschera PDO cheese. We focused on LAB which plays a critical role in the development of the unique characteristics of every cheese variety.

LAB microflora showed the highest count values among all microbial groups targeted, with values comparable to those reported for other italian ripened cheeses (Randazzo et al., 2002, 2006). LAB reached the highest levels in cheese samples after 3 days of salting and 15 days of ripening. Then they started to decrease in aged cheeses as a result of autolysis and microbial competition events (Fox et al., 2004). Yeast population also showed considerable count values, while enterococci and CNC did not reach loads higher than 10^7 cfu/g. Coliforms decreased at the beginning of ripening process but, unexpectedly, increased in number in samples at 70 days of ripening and counts of 10^4–10^5 cfu/g were found in the final product.

According to other authors (Ampe et al., 1999; Randazzo et al., 2002; Ercolini et al., 2003) it is noteworthy that some of the used media were not very selective; in fact, on MRS agar plates lactococci and staphylococci isolates were also found together with lactobacilli. On the contrary, KAA medium showed a high selectivity level towards enterococci.

The incubation temperature used from other authors (Fortina et al., 2003; Torres-Llanez et al., 2006) to discriminate mesophilic and thermophilic microflora in dairy environments did not allow us to obtain a marked growth differentiation among LAB species isolated from Raschera PDO.

Two hundred and seventy-four LAB isolates obtained from Raschera PDO were grouped by using RSA analysis which proved to be a useful tool to obtain preliminary information on high numbers of LAB isolates. Then the results obtained from species-specific PCR highlighted that *L. lactis* subsp. *lactis* was the species most frequently isolated in Raschera PDO samples at all different ripening stages. This is in agreement with the high occurrence, detected in many European artisanal dairy products (Cogan et al., 1997), of wild *L. lactis* subsp. *lactis* as predominant species in cheese making. According to RAPD-PCR by using D8635 primer (Andrighetto et al., 2004; Cocolin et al., 2005; Psoni et al., 2007), *L. lactis* subsp. *lactis* isolates found in Raschera PDO showed high similarity. They resulted to belong to a quite homogeneous population; a correlation between genotyping and provenience or ripening stage was not possible.

Other lactococcal isolates found in Raschera PDO belonged to the species *L. lactis* subsp. *cremoris* and *L. garvieae*. The latter has been always considered an important agent of septicaemia in fish (Eldar et al., 1999) but, in the last years, researchers have found this species in various dairy sources together with *L. lactis* although in a lower proportion (Klijn et al., 1995; Morea et al., 1999;

Villani et al., 2001; Blaiotta et al., 2002; Fortina et al., 2003, 2007); thus, a specific role of *L. garvieae* in dairy environment has been hypothesized (Fortina et al., 2007).

E. faecium, *E. faecalis* and *E. casseliflavus* were also isolated from Raschera PDO samples. The enterococci are part of the natural flora in cheese manufactured with raw milk and are believed to contribute to the ripening process and the development of flavour and aroma (Cogan et al., 1997). However, their presence in dairy samples is often associated to faecal contamination during milking and storing process (Garcia Fontan et al., 2001). *S. infantarius* subsp. *infantarius*, isolated from a few samples of Raschera PDO, is also considered an indicator of low hygienic conditions because it is a normal inhabitant of the rumen. Occasionally *V. carniphilus*, belonging to the group of vagococci, phylogenetically and phenotypically similar to enterococci (Shewmaker et al., 2004), was isolated. *Lc. paramesenteroides*, a common species in dairy environment (Callon et al., 2004; Florez et al., 2006) was also found in Raschera PDO.

In aged cheese samples heterofermentative lactobacilli belonging to the so-called "secondary culture" were also isolated. *Lb. paracasei* showed the highest frequencies while *Lb. plantarum* was occasionally found in few samples. Considerable amount of NS-LAB are commonly found in ripened semi-hard cheeses (Beresford et al., 2001) where they contribute to flavour development.

The results of the present research indicate that lactococci have a critical role in the manufacturing and ripening of Raschera PDO. In particular *L. lactis* subsp. *lactis* was found with the highest frequency values in all the samples analysed so that this species should be strongly considered in the selection of an adequate starter for the production of Raschera PDO.

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