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ORIGINAL RESEARCH

Genotypic and technological diversity of *Leuconostoc mesenteroides* and *Lactobacillus paracasei* subsp. *paracasei* strains for use as adjunct starter cultures in Pecorino di Filiano cheese

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Seventeen Leuconostoc mesenteroides *and 33* Lactobacillus paracasei *subsp.* paracasei *from traditional Pecorino di Filiano cheese were tested for their potential use as adjunct starters, and a study of their genetic variability was carried out. Forty one per cent (41%) of* Leu. mesenteroides *and 46% of* Lb. paracasei *subsp.* paracasei *showed medium–high proteolytic activity, while high lipolytic activity was detected in 18% of* Lb. paracasei *subsp.* paracasei. *The aminopeptidase activity of* Lb. paracasei *subsp.* paracasei *was higher than that of* Leu. mesenteroides. *Strain diversity by RAPD analysis showed a high degree of heterogeneity. This study identified strains with unusual properties that could be good candidates as adjuncts in a starter to manufacture PF cheese.*

Keywords Pecorino di Filiano, Adjunct culture, Technological features, Strain typing.

INTRODUCTION

Pecorino di Filiano (PF) is an artisanal cheese, produced in the north-western areas of the Basilicata region of Southern Italy and has a protected designation of origin (PDO) in compliance with the national legislation (EC 1992, 2007). PF is a semihard or hard cheese made from raw ewe's milk using traditional techniques and without the addition of selected starter cultures. The ripening process depends entirely on the indigenous microbial populations present in the milk and from the environment which promote a complex series of biochemical reactions very important for the development of flavour and texture (Bonomo and Salzano 2012). The microflora of cheese may be divided into two groups: starter lactic acid bacteria and secondary flora. Starter lactic acid bacteria are involved in acid production during manufacture and contribute to the ripening process. They may be either blends of defined strains or, as in the case of many cheeses manufactured by traditional methods, composed of undefined mixtures of strains which are either added at the beginning of manufacture or are naturally present in the cheese milk. Secondary flora generally plays a significant role during ripening and contributes significantly to the specific characteristics of a particular cheese variety. The secondary flora is generally composed of complex mixtures of bacteria, yeasts and moulds, that are specific to particular cheese varieties and their proteolytic and lipolytic enzymes play a significant role in texture, flavour, chemical composition and quality of the final product. Secondary flora may be added in the form of defined cultures, but in many situations are composed of adventitious microorganisms gaining access to the cheese either from ingredients or the environment (Beresford *et al.* 2001; Rantsiou *et al.* 2008). Knowledge of the microflora evolution in a particular product, such as PF cheese, which is confined to a well-defined geographical area with traditional manufacturing and ripening process, is important to protect the microbial biodiversity of this traditional cheese, and it is essential in dealing with product quality features, authenticity assurance and traceability along the entire production chain (Rantsiou *et al.* 2008). During ripening, in the artisanal PF cheese a complex natural microbial population has been observed, revealing the strong selective effect of the stringent typical conditions of the ripening environment and how a particular biological ecosystem affects and produces the typical

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features of an artisanal product. For distinct varieties of cheese, a predominance of secondary flora during ripening has been reported. Mesophilic lactobacilli are among the most common groups in this microbiota, although pediococci, micrococci and leuconostoc are also present in lower proportions. An important role in ripening has been attributed to these minority genera (Manolopoulo *et al.* 2003; Nieto-Arribas *et al.* 2010). In different cheeses, *Lactobacillus paracasei* subsp. *paracasei* was frequently isolated during manufacture, and *Leuconostoc mesenteroides* represent the species widely recognised for its role in the formation of aroma and texture and in the determination of the unique features of the final product (Cibik *et al.* 2001; Hemme and Foucaud-Scheunemann 2004).

The aim of this study was to characterise, on the basis of their genotypic and technological properties, different strains isolated from artisanal PF cheese to select appropriate strains with excellent properties to be introduced as adjunct cultures in the manufacture of this cheese. As a result, it should be possible to more closely reproduce the flavour of this product and contribute to the preservation of the indigenous microbial population responsible for the typical features of this important variety of cheese.

MATERIALS AND METHODS

Bacterial strains and growth conditions

A total of 50 strains, seventeen belonging to *Leu. mesenteroides* subsp. *mesenteroides* and 33 to *Lb. paracasei* subsp. *paracasei*, previously isolated and identified in the traditional Pecorino di Filiano cheese of Basilicata region (Bonomo and Salzano 2012), were used in this study. All strains were maintained as freeze-dried stocks in reconstituted (11% w/v) skim milk, containing 0.1% w/v ascorbic acid (Riedel-de Haën, Sigma-Aldrich, Milan, Italy) and routinely cultivated in MRS broth.

Technological characterisation

Proteolytic activity

The proteolytic activity of each strain was evaluated according to Perez *et al.* (2003) by using selective calcium caseinate agar medium. All strains were revitalised in MRS broth by overnight culture at 30 °C. Calcium caseinate agar medium was inoculated (1% (v/v)) with revitalised strains, distributed into plates and incubated at 20 °C for 72 h. After incubation, plates were sprayed with 10% of acetic acid solution. A clear zone surrounding the colonies, after one minute of exposure, indicated the proteolytic activity. The experiment was performed in triplicate, and the proteolytic activity was expressed in terms of the mean of diameter of clear zone (in mm) and was considered low (diameter < 8), middle (8 < diameter < 11) and high (diameter > 11).

Lipolytic activity

The lipolytic activity of each strain was determined according to the method described by Perez *et al.* (2003) using trybutyrin agar medium. The medium was inoculated (1% (v/v)) with revitalised strains, distributed into plates and incubated at 20, 25 and 30 °C for 72 h. The strains were considered positive when showing a clear zone surrounding colonies. The experiment was performed in triplicate, and the lipolytic activity was expressed in terms of the mean of diameter of clear zone (in mm) and was considered low (diameter < 10), middle (10 < diameter < 14) and high (diameter > 14).

Diacetyl production

The production of diacetyl from citrate was assessed in Elliker broth added with 0.5% w/v trisodium citrate, by using the Voges-Proskauer (VP) test as described by Perez *et al.* (2003). After incubation for 7 days at 30 °C, solutions of 5% alcoholic α-naphthol, 40% KOH and creatinine crystals were added to the tubes. Positive reactions were recorded when a colour change from yellow to red/pink appeared.

Peptidase activity of intact cells

The aminopeptidase (AP) activity was assayed using L-lysine-ρ-nitroanilide (Lys-ρNA) and L-leucine-ρ-nitroanilide (Leu-ρNA) as substrates, according to Nieto-Arribas *et al.* (2010). After incubation, the absorbance at 410 nm was measured using a UV-Vis Beckman DU-65 spectrophotometer. The results were expressed as aminopeptidase activity units, where 1 U corresponds to an increase in absorbance of 0.001 in 1 min.

Production of biogenic amines (BA)

The potential to produce the biogenic amines tyramine, histamine, putrescine and cadaverine was assayed by adding 1% (w/v) of each precursor amino acid, tyrosine disodium salt, L-histidine monohydrochloride, L-ornithine monohydrochloride and L-lysine on MRS agar plates containing 0.06% (w/v) bromocresol purple. Positive reactions were recorded when a purple colour appeared on the plates or tyrosine precipitates disappeared around the colonies as described by Bover-Cid and Holzapfel (1999).

Dextran production

Dextran production was tested by spotting 5 μL of an overnight culture on Rogosa plates and incubating at 25°C for 5 days, as described by Parente *et al.* (2001). All tests were carried out in duplicate, and the diameter of the mucous colonies was measured after incubation.

Strain typing

The strains were subjected to the total DNA extraction from a single colony using the IstaGene™ Matrix (Bio-Rad Laboratories Hercules, Berkeley, CA, USA) following the supplier's instruction. For screening of strains of each

species, one hundred nanograms of the DNA was submitted to RAPD analysis using primer M13 (5′-GAGGGTGGC GGTTCT-3′) as previously described by Bonomo *et al.* (2008). PCR amplification was carried out in a Genius Techne Progene thermal cycler (Cambridge, UK) using the following programme described by Mannu *et al.* (1999): initial denaturation at 90 °C for 10 min; 40 cycles of denaturation at 94 °C for 1 min, annealing at 45 °C for 20 s and extension at 72 °C for 54 s; ending with a final extension at 72 °C for 2 min. The PCR products were separated by electrophoresis on 2% (w/v) agarose gels at 100 V for 4 h. Gels were stained with 0.5 μg/mL ethidium bromide (Serva) for 30 min. A 1 kb DNA ladder (EuroClone) was used as molecular weight and normalisation gel standard.

Statistical analysis

All statistical analyses for technological characterisation were performed using Systat 10.0 for Windows (SPSS, Chicago, IL, USA), and statistical differences between the strains of a single bacterial species were determined by ANOVA analysis ($P < 0.05$). The strains were tested in triplicate for each technological feature. The banding patterns were visualised by UV trans-illumination and captured with a GelDoc 2000 Apparatus (Bio-Rad). Gel images were digitised in Diversity Database™ software (Bio-Rad Laboratories Ltd., Watford, Herts, UK) and processed for detection of the bands. Calculation of similarity in the profiles of RAPD bands was based on the Pearson product–moment correlation coefficient. Dendrograms were obtained by means of the unweighted pair group method using arithmetic average (UPGMA) clustering algorithm (Vauterin and Vauterin 1992). A coefficient of correlation of 90% was arbitrarily selected to distinguish the clusters.

Reagents and media

Unless otherwise specified all reagents were obtained from Sigma-Aldrich (Milan, Italy), while bacteriological media and ingredients were obtained from Oxoid (Basingstoke, Hampshire, UK).

RESULTS

Technological characterisation

The characterisation of strains on the basis of different activities and technological features was carried out to evaluate their potential use as indigenous starter cultures and to contribute to the protection of the indigenous microbial biodiversity responsible for the typical features of this important traditional cheese.

The proteolytic activity assay showed 41.2% of *Leu. mesenteroides* strains proved a medium high level (clear zone diameter ranging from 9.96 to 12.88 mm), with strains H65 and G71 showing the highest activity followed by H66, H58, H74 G55 and R12 strains (Table 1). Among *Lb. paracasei* subsp. *paracasei* strains, 45.5% presented a medium–high proteolysis with the highest values in V23, V58, M9, V28 and V13 strains, ranging from 8.52 to 12.68 mm (Table 1). The results shown in Table 1 indicate that there were significant differences ($P < 0.05$) in proteolytic activity among strains, even within the same species. Data are shown in Table 1 for the lipolytic activity of strains tested at three different temperatures. The highest lipolytic activity was detected at 20 °C for 18.2% of *Lb. paracasei* subsp. *paracasei* with the best response ranging from 13.56 to 18.02 mm, in U48, U56, M9 and V25 strains. At the same temperature, 35.3% of *Leu. mesenteroides* showed a middle lipolysis with the best values of 11.78 and 13.47 mm in G34 and H58 strains, respectively. An increase in temperature produced a slightly lower lipolysis in all strains, with a increase in the percentage of strains with medium ability, in *Leu. mesenteroides* of 17.6% and 11.8% and in *Lb. paracasei* of subsp. *paracasei* 18.2% and 9.1%, respectively, at 25 and 30 °C.

Moreover, the evaluation of diacetyl production from citrate revealed that most of *Leu. mesenteroides* and *Lb. paracasei* subsp. *paracasei* strains (76.5% and 87.9% respectively) were able to produce diacetyl from citrate (Table 1).

The assay for aminopeptidase activity using ρ-nitroanilide substrates (Leu-ρNA and Lys-ρNA) showed significant differences among strains, also in the same species (Table 1). For all *Leu. mesenteroides* strains, the lys-aminopeptidase activity was higher than leu-aminopeptidase activity; 35.3% showed high aminopeptidase activity with strain H65 exhibiting the highest activity towards both substrates followed by G71, R12 and G55, while 23.5% and 41.2% medium and low activity, respectively.

All *Lb. paracasei* subsp. *paracasei* strains demonstrated more leu-aminopeptidase activity than lys-aminopeptidase activity and strains M9 and V28 presented the highest values for both aminopeptidases, followed by V23 and V58 strains. The aminopeptidase activity of *Lb. paracasei* subsp. *paracasei* strains was higher than for *Leu. mesenteroides*, with good activity apparent for most of the strains.

Results for BA production by the fifty strains analysed showed that none of the *Leu. mesenteroides* strains was able to decarboxylate any of the four amino acids assayed, while most of *Lb. paracasei* subsp. *paracasei* strains (66.7%) were able to decarboxylate only L-tyrosine (Table 1). Only *Leu. mesenteroides* strains produced the characteristic slimy colonies corresponding to dextran producers, none of *Lb. paracasei* subsp. *paracasei* strains were able to do it.

Strain typing

RAPD PCR analysis was used to explore the genetic diversity of selected strains belonging to *Leu. mesenteroides* and *Lb. paracasei* subsp. *paracasei* species from PF artisanal cheese. The different banding patterns obtained by RAPD fingerprinting for the strains of each species tested were analysed by selecting arbitrarily a coefficient of correlation to distinguish the clusters. Numerical elaboration of the

Table 1 Proteolytic, lipolytic, aminopeptidase and decarboxylate activities and ability to produce diacetyl from citrate of each strain tested

Species	*Strain*	*Proteolytic activity*[a]	*Lipolytic activity*[b]			*Aminopeptidase activity*[c]		*Decarboxylation*[d]					*Diacetyl production*[e]
			20 °C	*25 °C*	*30 °C*	*Leucine*	*Lysine*	*His*	*Orn*	*Tyr*	*Lys*	*Phe*	
Leuconostoc	R39	NA	NA	8.96 ± 0.35	7.94 ± 1.23	0.03 ± 0.05	0.15 ± 0.03	0	0	0	0	0	1
mesenteroides	G34	6.42 ± 0.99	11.78 ± 0.66	10.87 ± 0.28	7.16 ± 1.22	0.23 ± 0.02	0.25 ± 0.05	0	0	0	0	0	1
subsp.	H12	7.58 ± 1.07	7.49 ± 0.42	NA	6.93 ± 0.88	0.01 ± 0.01	0.31 ± 0.04	0	0	0	0	0	1
mesenteroides	H13	7.67 ± 0.93	NA	NA	10.58 ± 1.07	0.18 ± 0.03	0.37 ± 0.02	0	0	0	0	0	0
	H56	5.99 ± 1.10	7.78 ± 0.66	9.87 ± 1.02	9. 64 ± 0.43	0.09 ± 0.02	0.47 ± 0.04	0	0	0	0	0	1
	H66	10.78 ± 0.89	NA	8.60 ± 0.72	8.83 ± 0.79	1.12 ± 0.03	1.67 ± 0.02	0	0	0	0	0	1
	H65	12.86 ± 0.23	7.47 ± 0.22	6.89 ± 0.88	NA	1.74 ± 0.09	2.76 ± 0.11	0	0	0	0	0	1
	H58	10.32 ± 1.03	13.47 ± 0.77	12.32 ± 0.53	9.88 ± 0.77	1.02 ± 0.08	2.08 ± 0.21	0	0	0	0	0	0
	H74	9.96 ± 0.35	6.93 ± 0.79	NA	NA	0.78 ± 0.03	1.05 ± 0.13	0	0	0	0	0	1
	H76	NA	NA	NA	NA	0.04 ± 0.01	0.13 ± 0.02	0	0	0	0	0	1
	H72	NA	NA	NA	12.67 ± 0.93	0.13 ± 0.11	0.83 ± 0.07	0	0	0	0	0	1
	G55	10.87 ± 0.28	12.79 ± 0.57	9.47 ± 0.77	NA	1.15 ± 0.09	2.03 ± 0.12	0	0	0	0	0	0
	G71	12.88 ± 0.77	NA	NA	NA	1.74 ± 0.02	1.98 ± 0.21	0	0	0	0	0	1
	R29	NA	NA	NA	NA	0.05 ± 0.04	0.22 ± 0.06	0	0	0	0	0	1
	R56	6.82 ± 0.53	11.64 ± 0.61	8.53 ± 0.86	NA	0.98 ± 0.11	1.23 ± 0.09	0	0	0	0	0	0
	R21	NA	NA	NA	NA	0.69 ± 0.02	0.88 ± 0.06	0	0	0	0	0	1
	R12	10.11 ± 0.12	13.78 ± 0.36	11.98 ± 0.66	NA	1.28 ± 0.12	2.53 ± 0.08	0	0	0	0	0	1
Lactobacillus	U15	10.86 ± 0.23	9.86 ± 0.43	NA	NA	1.78 ± 0.05	1.12 ± 0.03	0	0	0	0	0	1
paracasei	U22	7.32 ± 0.28	15.16 ± 1.10	11.43 ± 0.58	NA	0.97 ± 0.04	0.65 ± 0.05	0	0	1	0	0	1
subsp.	F52	9.77 ± 1.03	8.54 ± 0.63	NA	7.49 ± 0.82	1.02 ± 0.02	0.78 ± 0.01	0	0	0	0	0	1
paracasei	U45	NA	NA	NA	NA	0.18 ± 0.01	0.04 ± 0.03	0	0	0	0	0	1
	F70	NA	NA	6.37 ± 0.51	NA	0.76 ± 0.03	0.05 ± 0.04	0	0	0	0	0	1
	V52	7.42 ± 0.99	NA	11.35 ± 1.07	11.51 ± 0.96	1.22 ± 0.11	0.34 ± 0.03	0	0	1	0	0	1
	U52	6.88 ± 0.07	11.46 ± 0.23	9.73 ± 0.43	8.55 ± 0.62	1.16 ± 0.12	0.56 ± 0.04	0	0	1	0	0	1
	U50	7.77 ± 0.55	NA	7.99 ± 1.12	7.44 ± 0.84	1.23 ± 0.11	0.98 ± 0.09	0	0	0	0	0	1
	U48	6.99 ± 0.71	18.02 ± 1.03	16.08 ± 0.89	8.63 ± 0.58	2.43 ± 0.12	1.70 ± 0.22	0	0	1	0	0	1
	U53	10.31 ± 0.47	NA	9.58 ± 0.74	10.93 ± 0.78	3.45 ± 0.18	2.33 ± 0.09	0	0	1	0	0	1
	V49	10.76 ± 0.53	NA	NA	NA	2.86 ± 0.23	1.89 ± 0.08	0	0	1	0	0	1
	U42	5.74 ± 0.53	15.41 ± 0.99	9.93 ± 0.88	NA	2.66 ± 0.21	2.03 ± 0.11	0	0	1	0	0	1
	U25	5.68 ± 0.90	NA	9.50 ± 0.66	8.76 ± 0.45	0.87 ± 0.02	0.05 ± 0.01	0	0	0	0	0	1
	M71	6.76 ± 0.74	9.53 ± 0.62	9.11 ± 0.12	NA	1.01 ± 0.09	0.03 ± 0.02	0	0	0	0	0	0
	M73	10.88 ± 1.02	NA	NA	NA	2.04 ± 0.22	0.77 ± 0.09	0	0	1	0	0	1
	U68	6.74 ± 0.88	11.05 ± 1.06	NA	NA	1.86 ± 0.12	0.09 ± 0.01	0	0	0	0	0	1
	U79	NA	7.72 ± 0.38	8.06 ± 0.44	NA	1.16 ± 0.07	0.88 ± 0.02	0	0	0	0	0	1
	U80	9.79 ± 0.67	9.65 ± 0.98	9.53 ± 0.74	9.78 ± 0.88	3.09 ± 0.31	1.44 ± 0.22	0	0	1	0	0	1
	U56	7.75 ± 0.43	17.16 ± 1.22	12.48 ± 0.99	9.53 ± 0.67	3.46 ± 0.33	1.77 ± 0.21	0	0	1	0	0	1
	V23	12.02 ± 1.07	9.97 ± 0.37	15.48 ± 0.90	NA	6.35 ± 0.23	4.22 ± 0.33	0	0	1	0	0	1
	U73	10.32 ± 0.23	11.66 ± 0.92	9.42 ± 0.33	8.87 ± 0.91	4.86 ± 0.14	2.67 ± 026	0	0	1	0	0	1
	V58	11.31 ± 0.45	9.97 ± 0.37	9.73 ± 0.47	NA	6.16 ± 0.41	3.13 ± 0.18	0	0	1	0	0	1
	M9	12.68 ± 0.73	13.56 ± 1.04	16.42 ± 0.99	12.18 ± 0.22	7.68 ± 0.34	4.46 ± 0.19	0	0	1	0	0	0
	U26	NA	NA	NA	NA	5.59 ± 0.32	1.56 ± 0.12	0	0	1	0	0	0
	V28	12.36 ± 0.85	NA	NA	NA	7.34 ± 0.35	4.67 ± 0.26	0	0	1	0	0	1
	V26	7.55 ± 0.84	7.16 ± 0.32	10.69 ± 1.21	NA	0.78 ± 0.02	0.03 ± 0.01	0	0	1	0	0	1

Table 1 (Continued)

Species	Strain	Proteolytic activity[a]	Lipolytic activity[b]			Aminopeptidase activity[c]		Decarboxylation[d]				Diacetyl production[e]
			20 °C	25 °C	30 °C	Leucine	Lysine	His	Orn	Tyr	Lys	Phe	
	F6	9.86 ± 0.56	15.03 ± 1.02	NA	NA	1.39 ± 0.08	0.55 ± 0.01	0	0	1	0	0	0
	V13	11.85 ± 1.13	8.09 ± 0.65	NA	7.42 ± 0.36	5.16 ± 0.25	1.66 ± 0.22	0	0	0	0	0	1
	V29	NA	6.72 ± 0.56	6.68 ± 0.88	NA	2.03 ± 0.23	0.96 ± 0.09	0	0	0	0	0	1
	V40	9.02 ± 0.55	7.52 ± 0.54	7.04 ± 0.76	NA	3.22 ± 0.21	1.79 ± 0.11	0	0	1	0	0	1
	V51	7.88 ± 0.45	6.99 ± 0.31	10.81 ± 0.11	NA	2.46 ± 0.33	0.87 ± 0.04	0	0	1	0	0	1
	V25	7.90 ± 0.98	14.64 ± 1.15	11.58 ± 0.23	9.73 ± 0.55	3.22 ± 0.23	1.56 ± 0.12	0	0	1	0	0	1
	V31	8.52 ± 0.66	NA	NA	NA	1.99 ± 0.10	0.89 ± 0.01	0	0	1	0	0	1

NA: no activity was observed.

[a]Clear zone diameter <8 mm: low proteolytic activity; 8 < clear zone diameter < 11: middle proteolytic activity; clear zone diameter > 11: high proteolytic activity.

[b]Clear zone diameter <10 mm: low lipolytic activity; 10 < clear zone diameter < 14: middle lipolytic activity; clear zone diameter > 14: high lipolytic activity.

[c]Expressed as aminopeptidase activity units. 1 U was the amount of enzyme giving an absorbance increase of 0.001 units at 410 nm in 1 min.
Results are expressed as mean values ± standard deviation for each strain.

[d]Ability (1) or inability (0) of strains to decarboxylate amino acids.

[e]Ability (1) or inability (0) of strains to produce diacetyl from citrate.

different strains profiles resulted in the dendrograms shown in Figures 1 and 2. Figure 1 shows the profiles of *Leu. mesenteroides* strains; the lowest similarity level was 69%; hence, only clusters with a correlation coefficient above 69% were considered different. Cluster analysis of the banding patterns revealed two major clusters, A and B, with 83.5% similarity. Cluster A grouped four strains, while cluster B included ten strains and, moreover, at this similarity level, there was a single strain (G34) and a minor cluster of two strains (G55 and R21). As the use of RAPD method for genetic variability study of closely related isolates could reveal an agreement with technological groups and support the selection of strains with interesting properties to be used as adjunct cultures, a higher level of similarity was used to discriminate between strains. At a similarity level of 90%, three clusters (I, II and III) were detected comprising four, three and six strains, respectively, while G55 and R21 strains, that clustered each other at 83.5% similarity, did not share any similarity at the 90% level with any other strains and formed single-strain clusters. Moreover, G34 strains did not cluster with any other except at a similarity level of 69% where it clustered with all other strains. The dendrogram in Figure 2 shows that all *Lb. paracasei* subsp. *paracasei* strains only clustered at a similarity level lower than 79%. Increasing the similarity level to 83%, we distinguished a cluster A with seven strains and a large cluster B that contained twenty-five strains. A similarity level of 90% was also chosen to discriminate different biotypes inside the *Lb. paracasei* subsp. *paracasei* species. Five clusters, I, II, III, IV and V, were identified with three, four, eight and 16 strains, respectively, while two strains (V23 and V52) formed single-strain clusters.

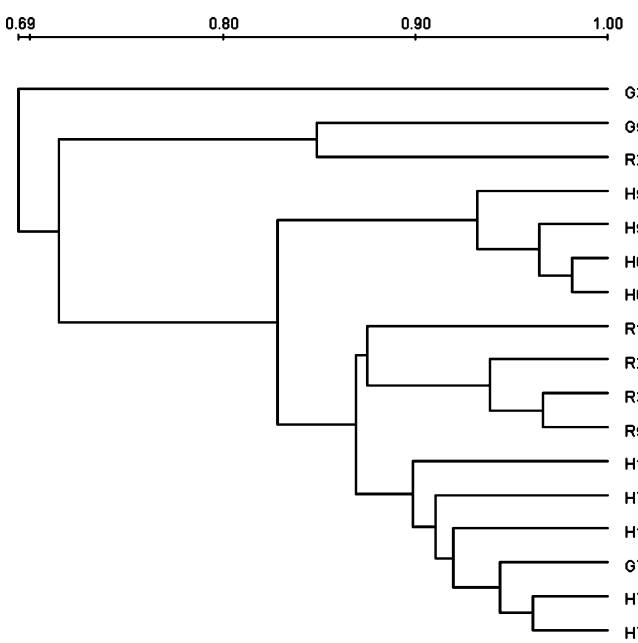

Figure 1 Cluster analysis of the profiles obtained by RAPD PCR from *Leuconostoc mesenteroides* strains used in this study.

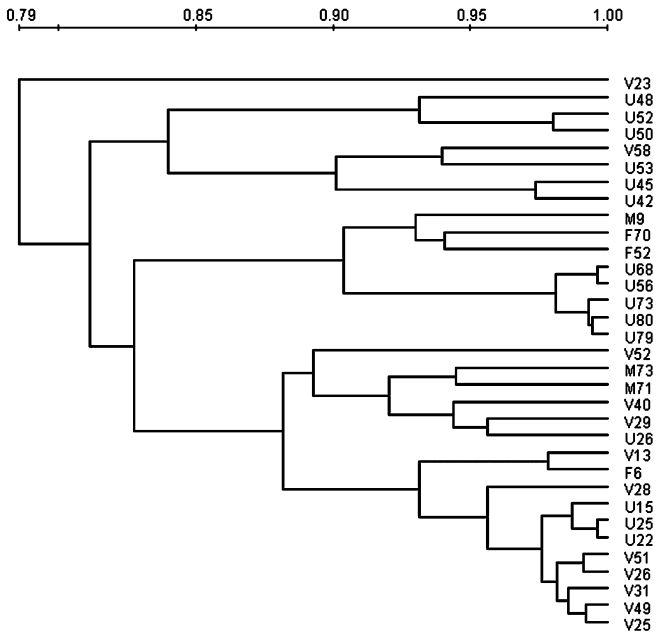

Figure 2 Cluster analysis of the profiles obtained by RAPD PCR from *Lactobacillus paracasei* strains used in this study.

DISCUSSION

In recent years, the accumulation of knowledge on the physiology of secondary flora during typical cheese manufacturing and ripening processes allows a better understanding of the composition and diversity of the indigenous bacterial population and of biochemical reactions essential for the development of flavour and texture. PF can be considered as a variety of cheese confined to a well-defined geographical area with deep-rooted traditions that protect the microbial biodiversity of such products with their quality, authenticity and traceability features, as already observed for other traditional cheeses (Rantsiou *et al.* 2008). In the present study, we characterised different isolates by using a combination of two approaches, based on technological and genotypic methods, to obtain a more effective strain differentiation. The proteolytic and lipolytic activities seem to be essential for autochthonous microflora in cheese, and they are involved in the development of some organoleptic characteristics in different fermented milk products (Christensen *et al.* 1999).

In our study, the proteolytic activity values were similar to those reported by other authors for *Leuconostoc* and *Lactobacillus* isolates (Herreros *et al.* 2003; Garabal *et al.* 2008; Nieto-Arribas *et al.* 2010). The proteolytic activity of *Leuconostoc* species has been described to be lower than that of lactococci, although differences between authors have been found when compared with lactobacilli. Herreros *et al.* (2003) and Garcia-Ruiz *et al.* (1997) obtained higher proteolytic activity for leuconostoc, while Ballesteros *et al.* (2006) found the opposite, and our results agreed with these last authors as *Leu. mesenteroides* strains showed a proteolytic activity slightly lower than that of *Lb. paracasei* subsp. *paracasei* strains. Several *Lb. paracasei* subsp. *paracasei* strains showed significant proteolytic activity when used as adjunct cultures in different cheese varieties (Hynes *et al.* 2001; Di Cagno *et al.* 2006; Bergamini *et al.* 2009). It has sometimes been described that the production of high-quality fermented dairy products is dependent on the proteolytic system of the starter bacteria used, because peptides and the amino acids formed have a direct impact on flavour or serve as flavour precursors (Nieto-Arribas *et al.* 2010). Proteolytic activity is a significant property for adjunct cultures because it can influence the background flavour of the product, providing most of the precursors of the aroma. However, it is important to bear in mind that highly proteolytic strains are not always the most suitable for use as starter cultures, because excessive proteolysis can cause uncontrolled production of bitter peptides and other undesirable compounds, or even excessive casein hydrolysis resulting in a final product that is too soft (Nieto-Arribas *et al.* 2009).

The low lipolytic activity, found in all strains tested, represents an important result and a useful contribution to aroma production, in agreement with other authors (Perez *et al.* 2003; Nieto-Arribas *et al.* 2009, 2010). This low activity in lactobacilli and leuconostoc strains is an important advantage, as the microflora normally occurring in cheese will result in the release of less free fatty acids from fat throughout ripening. Lactobacilli and leuconostoc strains used as starter cultures should ideally have low lipolytic activity, as the breakdown of milk fat during ripening should be slight to ensure the production of aroma without a rancid flavour (Nieto-Arribas *et al.* 2009).

The ability to produce diacetyl from citrate was observed in the most of *Leu. mesenteroides* and *Lb. paracasei* subsp. *paracasei* strains, and this result was in agreement with other authors as regards the lactobacilli strains (Perez *et al.* 2003; Nieto-Arribas *et al.* 2010). As regards leuconostoc strains, some authors observed that leuconostoc strains studied produced little acetoin or butanediol from citrate (Nieto-Arribas *et al.* 2010), while Perez *et al.* (2003) found that none of the leuconostoc strains from Tenerife cheese were able to produce diacetyl from citrate and explained that this ability is unstable in lactic acid bacteria because the citrate permease gene is plasmid linked. In this study, *Leu. mesenteroides* strains showed low leu- and lys-aminopeptidase activities as compared with the values reported by other authors (Macedo *et al.* 2000; Ayad *et al.* 2004; Aquilanti *et al.* 2007; Nieto-Arribas *et al.* 2010). A good aminopeptidase acitivity was observed for all *Lb. paracasei* subsp. *paracasei* strains, with leu-aminopeptidase activity higher than lys-aminopeptidase activity, and this is important as aminopeptidases play a key role in the hydrolysis of bitter peptides and flavour formation during cheese ripening (Nieto-Arribas *et al.* 2009). Other authors (Nieto-Arribas *et al.* 2009) reported similar results, while others (Macedo *et al.* 2000) have reported higher lys-

aminopeptidase values than those of leu-aminopeptidase for lactobacilli isolated from yoghurt and cheese starters.

As regards the amino biogenic capacity, most of the *Lb. paracasei* subsp. *paracasei* strains have been described as being able to decarboxylate L-tyrosine according to other authors (Bover-Cid and Holzapfel 1999; Roig-Sagues *et al.* 2002; Madera *et al.* 2003; Nieto-Arribas *et al.* 2009) who reported that tyramine is the BA produced by the largest number of lactic acid bacteria and therefore the most abundant in fermented foods. Moreover, our results are in agreement with Bover-Cid and Holzapfel (1999) and Nieto-Arribas *et al.* (2010) who reported that *Leuconostoc* spp. showed no decarboxylate activity. However, it is important to highlight that negative results for amine production in the laboratory do not necessarily imply a similar behaviour in cheese. Some authors (Joosten and van Boekel 1988; Nieto-Arribas *et al.* 2010) reported that factors such as pH, NaCl concentration, availability of substrate, etc., can influence the build-up of biogenic amines.

In dairy technology, the production of dextran or other exopolysaccharides is considered to be a relevant feature for lactic acid bacteria used as starters because they act as texturisers and stabilisers, and smooth creamy products have considerable appeal for consumers. In our study, all the *Leu. mesenteroides* strains tested showed this property that recently is receiving increasing attention because exopolysaccharides-producing lactic acid bacteria have been recognised as generally recognized as safe (GRAS) bacteria (Vijayendra *et al.* 2008).

The data obtained by the RAPD fingerprinting technique showed the presence of different biotypes within the same species, demonstrating the strong selective effect of the stringent typical conditions of the cheese manufacturing on the indigenous microflora. These conditions can determine the selection of specific microbial populations and also of particular strains of each bacterial group (Bonomo *et al.* 2008). The combination of genotypic and technological results established that the RAPD clusters obtained were composed not only of different genotypes but of different technological profiles too. Major heterogeneity was observed with *Leu. mesenteroides* strains because within each cluster, different technological profiles were detected and also the four single strains presented four different technological features. The different degree of heterogeneity in the bacterial species studied by cluster analysis of the banding patterns obtained, suggested the importance of some species during the production process and that the typical characteristics of the products are due, at least partially, to the presence of different strains. Moreover, with respect to proteolytic and lipolytic activities, biotypes characterised by a different behaviour, as well as aminopeptidase activity, were observed. Among the strains assayed, *Leu. mesenteroides* and *Lb. paracasei* subsp. *paracasei* strains exhibited particularly interesting technological features that make them good candidates for inclusion in an adjunct culture.

The intraspecific biodiversity evaluation of the tested strains and the discrimination based on analysis of technological features allowed us to conclude that strains G55, H58 and R12, belonging to *Leu. mesenteroides* and strains V23, M9, U73 and U80, belonging to *Lb. paracasei* subsp. *paracasei*, could be selected to be included as adjunct starter cultures, because they fulfilled the customary requirements for cheesemaking. The choice of strains with important technological characteristics has been done to test and to compare their abilities in the manufacture of PF cheese. The strains G55 and H58 of *Leu. mesenteroides* were chosen for their good proteolytic, lipolytic and aminopeptidase activities and because G55 was a single strain in the RAPD dendrogram, while H58 belonged to cluster I with three other strains but it possess properties different than the others. Moreover, strain R12 showed medium–high proteolytic, lipolytic and aminopeptidase activities, a good ability to produce diacetyl from citrate and also it was a single strain of the RAPD analysis. The strains M9, U73 and U80 of *Lb. paracasei* subsp. *paracasei*, even if they belong to the same RAPD cluster (III), were chosen because they showed different but interesting technological features. M9 had a high level of proteolytic, lipolytic and aminopeptidase activities, the ability to decarboxylate tyrosine but it was not able to produce diacetyl from citrate, while U73 and U80 had medium proteolytic and lipolytic activities and the ability to decarboxylate tyrosine and to produce diacetyl. The two strains were differentiated on the basis of aminopeptidase activity, medium level for U73 and low for U80. Moreover, V23 was chosen for its good technological features and because it was a single strain of the RAPD analysis.

CONCLUSIONS

From the results presented in this study, we concluded that PF cheese is a very complex microbial ecosystem with a very complex microflora characterised by a species–site dominance, and this dominance is closely related to the environmental parameters. Traditional cheeses show a distinctive organoleptic profile, and this can be explained by admitting that the isolated strains, although they belong to the species commonly regarded as responsible for ripening process, possess specific physiological and technological characteristics that make these traditional products unique. Therefore, the microflora plays a major role in cheese ripening, and the selection of suitable strains would enable the cheese maker to control or modify flavour development. The technological characterisation and the investigation of strain variability of specific bacterial strains were useful means of obtaining atypical strains with unusual properties, which can contribute to the quality and the development of typical PF cheese taste and flavour. This work can be considered a preliminary study in developing an adjunct starter that could be used to favour the protection of the natural microflora and the preservation of typical features in the production of this important variety of traditional cheese. Further research is required to determine

how these adjuncts behave in combination and with the starter strains, and also to investigate the behaviour of these strains in the cheese environment to determine their growth dynamics and the influence on flavour formation during ripening.

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