Genotypic and technological diversity of Brevibacterium linens *strains for use as adjunct starter cultures in 'Pecorino di Filiano' cheese ripened in two different environments*

Maria Grazia Bonomo, Caterina Cafaro & Giovanni Salzano

Folia Microbiologica
Official Journal of the Institute of Microbiology, Academy of Sciences of the Czech Republic and Czechoslavak Society for Microbiology

ISSN 0015-5632
Volume 60
Number 1

Folia Microbiol (2015) 60:61-67
DOI 10.1007/s12223-014-0341-3

Springer

Folia Microbiol (2015) 60:61–67
DOI 10.1007/s12223-014-0341-3

Genotypic and technological diversity of *Brevibacterium linens* strains for use as adjunct starter cultures in 'Pecorino di Filiano' cheese ripened in two different environments

Maria Grazia Bonomo · Caterina Cafaro · Giovanni Salzano

Received: 20 March 2014 / Accepted: 6 August 2014 / Published online: 23 August 2014

Abstract Twenty-two *Brevibacterium linens* strains isolated from 'Pecorino di Filiano' cheese ripened in two different environments (natural cave and storeroom) were characterized and differentiated for features of technological interest and by genotypic methods, in order to select strains with specific features to be used as surface starter cultures. Results showed significant differences among strains on the basis of physiological and technological features, indicating heterogeneity within the species. A middle-low level of proteolytic activity was observed in 27.3 % of strains, while a small group (9.1 %) showed a high ability. Lipolytic activity was observed at three different temperatures and the highest value was detected at 20 °C with 13.6 % of strains, while an increase in temperature produced a slightly lower lipolysis in all strains. The evaluation of diacetyl production revealed that only 22.8 % of strains showed this ability, and most of them were isolated from product ripened in the natural cave. All strains exhibited only leu-aminopeptidase activity, with values more elevated in strains coming from the natural cave product. The combined analysis of genotypic results with the data obtained by the features of technological interest study established that the random amplified polymorphic DNA (RAPD) clusters obtained were composed not only of different genotypes but of different profiles based on technological properties too. This study demonstrated the importance of the ripening environment that affects the typical features of the artisanal product, leading to the selection of a specific surface microflora. Characterized strains could be associated within surface starters to standardize the production process of cheese, but preserving its typical organoleptic and sensory characteristics and improving the quality of the final product.

M. G. Bonomo (✉) · C. Cafaro · G. Salzano
Dipartimento di Scienze, Università degli Studi della Basilicata, Viale dell'Ateneo Lucano, 10, 85100 Potenza, Italy
e-mail: mariagrazia.bonomo@unibas.it

Introduction

Brevibacteria are industrially important microorganisms used in the manufacture of different types of cheeses. Their high proteolytic and varied lipolytic activities, aminopeptidase activity and diverse biochemistry and also their ability to produce volatile sulfur compounds raised great interest in their potential use as novel flavour adjuncts in the cheese manufacture (Lamberet et al. 1997; Weimer et al. 1997; Ummadi and Weimer 2001). Their importance for the surface of cheeses has often been stated, but relatively few studies have attempted to identify these bacteria (Rademaker et al. 2005). The microflora of smear surface-ripened cheese is generally complex and of a transient nature. Typically, ripening progresses from a yeast and mould flora to a bacterial flora, of which *Brevibacterium linens* is a major component (Rattray and Fox 1999). *B. linens* has long been recognized as an important dairy microorganism because of its ubiquitous presence on the surface of a variety of smear surface-ripened cheeses (Leclercq-Perlat et al. 2000; Hemme et al. 1982). The metabolism and physiology of *B. linens* influence the physical and chemical parameters of smear surface-ripened cheese and contribute to the typical feature formation and taste and texture development as well as to the complexity of the cheese ripening (Motta and Brandelli 2008). The major factors that influence the distinctive characteristics of smear surface-ripened cheeses and the number, type and growth rate of the surface microflora are the physical and chemical characteristics intrinsic to the cheese (pH, water activity, redox potential, composition and size), the ripening environment and its parameters (ripening temperature, relative humidity), the technological practices and the manufacturing and ripening conditions (washing of the cheese surface with brine, ripening time, degree of mechanization and microflora of cheese equipment) (Rattray and Fox 1999). 'Pecorino di Filiano' (PF) is a semi-hard or hard cheese, produced in north-western areas of

Basilicata region (Southern Italy) using traditional techniques and bearing a Protected Designation of Origin (PDO) in compliance with the national legislation (EC 2007). Since 1600, the ripening process is performed in natural tuff caves that give the freshness and unique features to the final product. A natural cave is a biological ecosystem with natural and not reproducible conditions due to the interaction and activities of cheese and environment microflora. The ripening process depends entirely on the indigenous microbial populations that originated from the milk and from the environment. The traditional manufacturing technology, applied for the production of PF according to its designation as a PDO cheese (EC 1992), points out that the peculiar characteristics of PF are strictly related to the geographical area of production, to the race and the nutrition of the ewes providing milk and particularly to the composition and diversity of the indigenous bacterial population and to its contribution to the biochemical and sensorial features (Gobbetti et al. 1997; Bonomo and Salzano 2012). Recently, the presence and the evolution of microbial populations during PF cheese ripening were investigated by comparing cheese samples manufactured with the same technology but ripened in two different environments (natural cave and storeroom) (Bonomo and Salzano 2012). Some microbial groups were present with no relevant differences in both ripening environments underlining the similar contribution of these groups to the final typical product, while the elevated presence of specific microbial groups evidenced the important selective effect of ripening conditions and the environment of the production area. Therefore, the ripening environment has a significant role in the growth and development of specific species and strains with peculiar biochemical traits, as shown for *B. linens* (Rattray and Fox 1999). The aim of the study was the characterization and the differentiation of *B. linens* strains isolated from a cheese ripened in two different environments (natural cave and storeroom) in order to select strains with specific features to be used as surface starters and to confirm that the bound to a well-defined geographical area with deep-rooted traditions is fundamental to protect the microbial biodiversity of typical products with their quality, authenticity and traceability features.

Materials and methods

Bacterial strains and growth conditions

Twenty-two strains of *B. linens*, previously identified and isolated from traditional 'Pecorino di Filiano' cheese of Basilicata region (Bonomo and Salzano 2012), were used in this study. All strains were isolated from the surface of the cheese divided into two batches, one placed in a natural cave and another in a storeroom with controlled temperature (12–14 °C) and relative humidity (80–85 %) for 120 days of ripening; the strains were collected after salting (t=0), at the mid-ripening (t=30 days) and at the end of ripening (t=120 days) (Table 1). All strains were maintained as freeze-dried stocks in reconstituted (11 % *w*/*v*) skim milk, containing 0.1 % *w*/*v* ascorbic acid and routinely cultivated in MRS broth at 30 °C for 16 h.

Physiological characterization

The strains were submitted to physiological and biochemical tests. They were examined for morphology, CO_2 production and ability to grow at 15 and 45 °C. Moreover, growth in the presence of 4 and 6 % NaCl, hydrolysis of arginine and esculin and dextran production were evaluated by miniaturized assays in microtiter plates. All tests were performed in triplicate.

Technological characterization

Proteolytic activity

The proteolytic activity of each strain was evaluated according to Bonomo and Salzano (2013) by using a selective calcium caseinate agar medium. The experiment was performed in triplicate, and the proteolytic activity was expressed in terms of the mean diameter of the clear zone (in mm) and was considered low (diameter<9), middle (9<diameter<13) and high (diameter>13).

Lipolytic activity

The lipolytic activity of each strain was determined according to the method described by Bonomo and Salzano (2013) using a trybutirin agar medium. The experiment was performed in triplicate, and the lipolytic activity was expressed in terms of the mean diameter of the clear zone (in mm) and was considered low (diameter<9), middle (9<diameter<13) and high (diameter>13).

Diacetyl production

The production of diacetyl from citrate was assessed in Elliker broth added with 0.5 % trisodium citrate, by using the Voges-Proskauer (VP) test as described by Bonomo and Salzano (2013).

Aminopeptidase 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 by a UV-Vis Beckman DU-65 spectrophotometer. The results were expressed as aminopeptidase activity

Table 1 Proteolytic, lipolytic and aminopeptidase activities, ability to produce diacetyl from citrate and RAPD cluster of *B. linens* strains used in this study

Brevibacterium linens strains	Proteolytic activity[a]	Lipolytic activity[b]			Aminopeptidase activity[c]	Diacetyl production[d]	Cluster number[e]
		20 °C	25 °C	30 °C	Leucine		
Surface cheese after salting (t=0)							
G3	5.57±0.99	NA	8.76±0.35	7.92±0.83	0.38±0.03	0	V
G4	NA	NA	8.87±0.28	7.16±1.22	0.04±0.01	0	I
G12	NA	7.49±0.42	NA	6.93±0.88	0.98±0.11	1	I
G11	6.79±0.93	14.78±1.16	12.43±0.73	10.58±1.07	1.05±0.09	0	*
Surface cheese at mid-ripening (t=30 days)							
Natural cave							
G41	14.58±1.07	14.47±0.22	12.89±0.88	11.32±0.53	3.46±0.33	0	IV
G42	11.67±1.10	11.79±0.57	9.47±0.77	NA	0.86±0.09	0	IV
G40	12.36±0.85	12.93±0.79	12.65±1.23	11.72±0.65	3.55±0.23	1	*
G43	12.45±0.78	12.84±0.89	12.58±0.44	11.97±0.86	4.86±0.14	1	*
G35	14.22±1.12	NA	9.83±0.36	9.67±0.93	1.86±0.44	0	V
G45	12.43±0.66	13.98±0.77	12.98±0.57	12.33±0.77	3.04±0.22	0	V
Store room							
G19	NA	8.72±0.81	NA	NA	0.45±0.18	0	V
G25	NA	NA	8.53±0.86	NA	0.69±0.07	0	V
G21	NA	NA	NA	NA	1.28±0.12	1	V
G16	11.52±0.89	12.78±0.36	12.98±0.66	12.64±0.43	3.39±0.32	0	III
G17	7.95±0.58	7.78±0.66	8.87±1.02	9.60±0.72	1.34±0.35	0	III
G26	NA	NA	NA	8.83±0.79	0.94±0.13	0	V
G27	8.12±0.46	8.67±0.74	8.36±0.63	NA	1.04±0.22	0	V
G30	6.88±0.97	8.42±0.59	7.87±0.87	NA	0.99±0.22	0	V
T16	NA	NA	9.86±0.75	9.74±0.33	1.88±0.12	0	V
Surface cheese at the end of ripening (t=120 days)							
Natural cave							
G62	6.76±0.98	11.64±0.61	9.69±0.38	NA	1.16±0.07	0	V
G72	10.89±0.77	12.86±0.97	12.65±1.03	11.92±0.85	3.09±0.31	1	II
G65	NA	NA	NA	9.66±0.89	0.94±0.35	0	II

All tests were carried out in triplicate

NA no activity was observed

[a] Clear zone diameter<9 mm, low proteolytic activity; 9 mm<clear zone diameter<13 mm, middle proteolytic activity; clear zone diameter>13 mm, high proteolytic activity

[b] Clear zone diameter<9 mm, low lipolytic activity; 9 mm<clear zone diameter<13 mm, middle lipolytic activity; clear zone diameter>13 mm, high lipolytic activity

[c] Expressed as aminopeptidase activity units. One unit 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 produce diacetyl from citrate

[e] Cluster number indicates the RAPD-PCR cluster to which strains belong

*single-strain cluster

units, where 1 U corresponds to an increase in absorbance of 0.001 in 1 min.

Strain typing

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

For screening of strains, 100 ng of the DNA was submitted to random amplified polymorphic DNA (RAPD) analysis using primer M13 (5′-GAGGGTGGCGGTTCT-3′) as described by Bonomo et al. (2008). The PCR mixture (25 μL) consisted of 1 μL of DNA, 2.5 μL of 1× PCR buffer

(EuroClone), 3.5 mmol/L of $MgCl_2$ (EuroClone), 0.4 mmol/L of each dNTP (EuroClone), 0.6 μmol/L of the primer (Invitrogen) and 2.5 U of *Taq* polymerase (EuroClone). 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 normalization gel standard. The analysis was performed in triplicate.

The banding patterns were visualized by UV transillumination and captured with a Gel Doc 2000 apparatus (Bio-Rad). Gel images were digitized 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.

Statistical analysis

All statistical analyses about technological characterization were performed using Systat 10.0 for Windows (SPSS, Chicago, IL, USA), and statistical differences between the strains of this species were determined by ANOVA ($p<0.05$). The strains were tested in triplicate for each test.

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, England).

Results

A characterization based on physiological features and properties of technological interest was carried out on the *B. linens* strains.

All strains were able to grow at 15 °C and this is fairly common since the ripening temperature is usually high at the beginning and then decreases to 12–14 °C during ripening till the end of the process, while the ability to grow at 45 °C was less frequent, with only the strains coming from the natural cave product being able to grow even at this temperature. Moreover, all *B. linens* strains tolerated high salt concentrations (4 and 6 %), while none of the strains either produced gas from glucose or were able to hydrolyze esculin and arginine and also none were able to produce the characteristic slimy colonies corresponding to dextran production (data not shown).

As shown in Table 1, the values of proteolytic activity showed that 27.3 % of *B. linens* strains, coming from the natural cave product, had a middle level with the clear zone diameter ranging from 10.89 to 12.45 mm, except for G62 with a low activity, while only a small group (9.1 %) showed a high ability, with strains G41 and G35 having a diameter of 14.58 and 14.22 mm, respectively. On the contrary, 27.3 % of *B. linens* strains coming from the storeroom product presented a low activity, except for G16 with a middle value of 11.52 mm (Table 1). These results indicate that there were significant differences ($p<0.05$) among the values of proteolytic activity for all strains. Of the strains, 13.6 % showed the highest (clear zone diameter ranging from 13.98 to 14.78 mm) lipolytic activity at 20 °C; the increase in temperature produced a slightly lower lipolysis in all strains, with middle ability in 50 % of strains. In particular, the strains with a better lipolytic activity were G11 coming from the surface sample after salting ($t=0$) and G41 and G45 from the natural cave mid-ripening product that showed a high ability at 20 °C and then a middle level at higher temperatures. Moreover, three strains from the natural cave sample (G40, G43, G72) and one from the storeroom product (G16) had a steady middle activity at all temperatures tested (Table 1).

The evaluation of diacetyl production revealed that only 22.8 % of *B. linens* strains showed this ability, and most of them were isolated from the product ripened in the natural cave (Table 1).

The assay for aminopeptidase activity using ρ-nitroanilide substrates (leu-ρNA and lys-ρNA) showed significant differences among strains, according to the ripening environment, as shown in Table 1. All *B. linens* strains exhibited no activity on the lysine substrate but only leu-aminopeptidase activity, and strains G40, G41 and G43 presented the highest values among others, followed by strains G45, G16 and G72. The aminopeptidase activity of *B. linens* strains coming from the natural cave product had values more elevated than those coming from the storeroom product with a good activity for most of them (Table 1).

RAPD-PCR analysis was used to explore the genetic diversity of selected strains, resulting in the dendrogram shown in Fig. 1. The banding pattern analysis depicted in the figure shows that the lowest similarity level obtained by repeated RAPD analysis was 66 %; hence, only clusters with a correlation coefficient above 66 % were considered different. An increase to 86.5 % of similarity allowed the discrimination of two minor clusters, A and B, of two strains each and two main clusters, C and D, that included 6 and 12 strains, respectively.

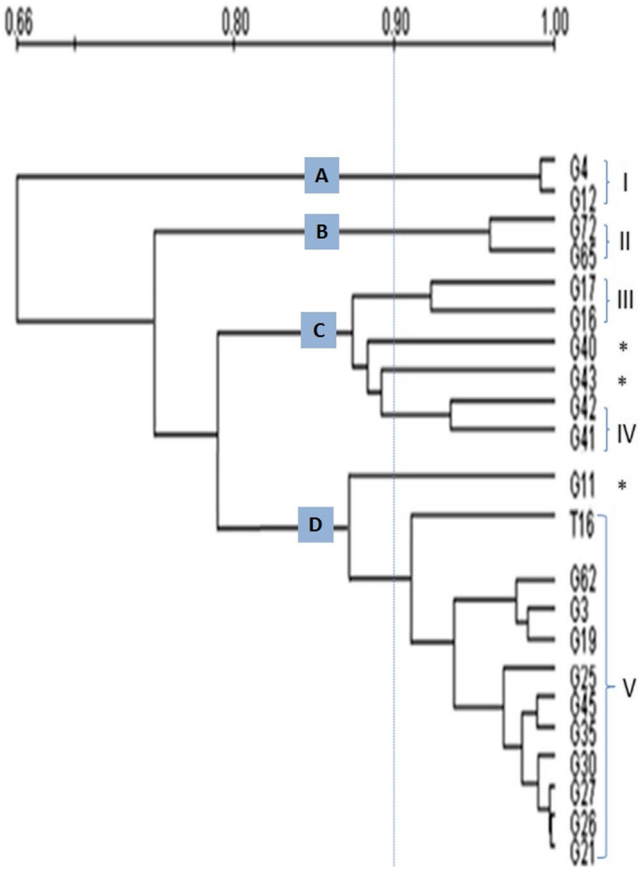

Fig. 1 Cluster analysis of the profiles obtained by RAPD-PCR from *B. linens* strains used in this study. *single-strain cluster

At a similarity level of 90 %, five clusters (I, II, III, IV and V) were detected while three strains formed unique patterns. Clusters I, II, III and IV were small clusters of two strains each, some closely related also above 95 % of similarity, while cluster V was a large cluster with 11 strains. Strains G40 and G43 clustered with four other strains in main cluster C at 86.5 % similarity, while at a similarity level of 90 %, they formed single-strain clusters. Moreover, strain G11 did not cluster with any other strain except at a similarity level of 86.5 %, in which it clustered with 11 other strains in cluster D.

Discussion

The genus *Brevibacterium* is a heterogeneous group of coryneform bacteria that have different applications, such as in cheese production (Rattray and Fox 1999; Amador et al. 1999), and most research has demonstrated that the physiology and metabolic activities of this bacterium are significantly strain-dependent. *B. linens* contributes to colour formation, proteolysis and lipolysis in smear surface-ripened cheeses (Motta and Brandelli 2008; Valdes-Stauber et al. 1997). It was recently shown that smear surface-ripened cheeses could be produced with a defined starter mix containing only a few microbial strains necessary to obtain the qualitative taste and texture characteristics of these cheeses (Bockelmann and Hoppe-Seyle 2001). *B. linens* was observed with an elevated frequency of isolation on the surface of the natural cave PF product with a constant presence during the whole ripening process, while the ripening in a storeroom led to a complete reduction of the species at the end of the process (Bonomo and Salzano 2012). This suggests the importance of the ripening environment of cheese and how a biological ecosystem, such as a natural cave, affects and produces the typical features of artisanal products.

In this study, different *B. linens* strains, by using a combination of two approaches, based on technological and genotypic methods, were characterized, to increase and obtain a more effective strain differentiation in order to select the strains more suitable to be used as appropriated surface cultures. *B. linens* is required commercially in the dairy industry for the production of smear surface-ripened cheeses to overcome the length of the ripening period, which is one of the most significant challenges facing the dairy and food industries in the area of cheese manufacture (Rattray and Fox 1999). The use as a cheese ripening accelerant and being responsible for the characteristic taste, aroma and colour of surface smear cheese mean that *B. linens* must contribute to the breakdown of lipids and proteins during cheese ripening by synthesizing highly active and multiple proteolytic and lipolytic enzymes during its growth, thus influencing the characteristic flavour of the ripened cheese (Motta and Brandelli 2008).

Physiological features and technological characteristics were investigated, and results showed significant differences among strains, indicating heterogeneity within the species in accordance with different previous studies as described by Rattray and Fox (1999). The assays showed that strains had good proteolytic and lipolytic activities in accordance with other studies (Rattray and Fox 1999) and the activities presented better values in strains coming from natural cave product, underlining again the influence and the effect of a natural ripening environment on the organoleptic characteristic development. As observed by other authors (Rattray and Fox 1999), a relationship between the proteolytic and lipolytic activities of *B. linens* strains was detected; strains with middle or strong proteolytic activity also had relatively high lipolytic activity. Moreover, as regards the aminopeptidase activity, all strains proved a strong preference for leucine with a good activity and no ability to hydrolyze lysine; also, in this case, strains with higher activity come from the natural cave product at the mid-ripening.

The data obtained by the RAPD fingerprinting technique showed the presence of different biotypes inside the *B. linens* species, evidencing the strong selective effect of the stringent typical conditions of the cheese manufacturing on the indigenous microflora, able to determine the selection of specific

microbial populations and also of particular strains of each bacterial group (Bonomo et al. 2008). The combined analysis of genotypic results with the data obtained by the study of technological interest features established that the RAPD clusters obtained were composed not only of different genotypes but of different profiles based on technological properties too. The major heterogeneity was observed inside cluster V in which different technological property-based profiles were detected and then the three single strains presented three different technological features. The different degrees of heterogeneity in the bacterial species studied by cluster analysis of banding patterns obtained suggested the importance of this species during the production process and, so, 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 characterized by a different behaviour, as well as aminopeptidase activity, were observed. The intraspecific biodiversity evaluation of the tested strains and the discrimination on the basis of technological feature analysis allowed the selection of strains suitable to be used as adjunct culture. The selection was performed holding in consideration that, even if proteolytic activity is a significant property of adjunct cultures since it can influence the background flavour of the product providing most of the precursors of the aroma, an excessive proteolysis can cause uncontrolled production of bitter peptides and other undesirable compounds and it could be influenced by different factors (Zemanovic et al. 1992). In this case, aminopeptidases play a key role in the hydrolysis of bitter peptides and flavour formation during cheese ripening. The low lipolytic activity, instead, was an important advantage, as the microflora normally occurring in cheese must have a reduced effect on the release of free fatty acids from fat throughout ripening (Nieto-Arribas et al. 2009, 2010).

On the ground of these considerations, we concluded that strains G40, G41, G43, G45, G72 and G16 could be selected to be included as adjunct surface cultures, since they fulfilled the customary requirements for cheesemaking. The choice of strains with important technological characteristics has been done in order to test and to compare in the next future their abilities in the manufacture of PF cheese. Strains G43, G40, G72 and G16 were chosen for their middle proteolytic and lipolytic activities and high aminopeptidase ability, while G45 was chosen for its middle proteolysis, middle-high lipolytic activity and high aminopeptidase ability and G41 for its high proteolytic and aminopeptidase activities and middle-high lipolytic ability. G40, G43 and G72 also showed a good ability to produce diacetyl from citrate. Moreover, besides different behaviours and activities, the selected strains belonged also to different RAPD clusters; G40 and G43 were single strains; G41, G16 and G72 belonged to the small clusters IV, III and II, respectively, in which each of them clustered with another strain that, however, possessed low/no activities; and G45, even if it belonged to the large cluster V with other ten strains, presented completely different but interesting and good technological features. It is very important to observe that all chosen strains came from the natural cave product, except one (G16) that came from the storeroom product.

In conclusion, the results of this study demonstrate that the physiology and metabolic activities of *B. linens* are significantly strain-dependent and also that the ripening environment and its specific conditions affect the bacterial properties producing the typical features of the artisanal product. This underlines the importance of the development of a native surface starter culture as a more suitable tool to standardize the production process of cheese, but preserving its typical organoleptic and sensory characteristics and improving the quality of the final product.

References

Amador E, Castro JM, Correia A, Martin JF (1999) Structure and organization of the rrnD operon of '*Brevibacterium lactofermentum*': analysis of the 16S rRNA gene. Microbiol 145:915–924

Bockelmann W, Hoppe-Seyle T (2001) The surface flora of bacterial smear-ripened cheeses from cow's and goat's milk. Int Dairy J 11: 307–314

Bonomo MG, Salzano G (2012) Microbial diversity and dynamics of Pecorino di Filiano PDO, a traditional cheese of Basilicata region (Southern Italy). Int J Dairy Tech 65:531–541

Bonomo MG, Salzano G (2013) Genotypic and technological diversity of *Leuconostoc mesenteroides* and *Lactobacillus paracasei* strains for use as adjunct starter cultures in 'Pecorino di Filiano' cheese. Int J Dairy Tech 66:402–409

Bonomo MG, Ricciardi A, Zotta T, Parente E, Salzano G (2008) Molecular and technological characterization of lactic acid bacteria from traditional fermented sausages of Basilicata region (Southern Italy). Meat Sci 80:1238–1248

EC (1992) European Council regulation no. 2081/92, July 14, 1992.

EC (2007) European Council regulation no. 1485/2007 of 14 December 2007, OJEU, L330 (2007), 13–14

Gobbetti M, Corsetti A, Smacchi E, De Angelis M, Rossi J (1997) Microbiology and biochemistry of Pecorino Umbro cheese during ripening. Italian J Food Sci 2:111–126

Hemme D, Bouillanne C, Metro F, Desmazeaud M (1982) Microbial catabolism of amino acids during cheese ripening. Sci Aliment 2: 113–123

Lamberet G, Auberger B, Bergere JL (1997) Aptitude of cheese bacteria for volatile S-methyl thioester synthesis I. Effect of substrates and pH on their formation by *Brevibacterium linens* GC171. Appl Microbiol Biotechnol 47:279–283

Leclercq-Perlat MN, Oumer A, Bergere JL, Spinnler HE, Corrieu G (2000) Behavior of *Brevibacterium linens* and *Debaryomyces hansenii* as ripening flora in controlled production of smear soft cheese from reconstituted milk: growth and substrate consumption. J Dairy Sci 83:1665–1673

Mannu L, Paba A, Pes M, Floris R, Scintu MF, Morelli L (1999) Strain typing among enterococci isolated from home-made Pecorino Sardo cheese. FEMS Microbiol Lett 170:25–30

Motta AS, Brandelli A (2008) Properties and antimicrobial activity of the smear surface cheese coryneform bacterium *Brevibacterium linens*. Eur Food Res Technol 227:1299–1306

Nieto-Arribas P, Poveda JM, Sesena S, Palop L, Cabezas L (2009) Technological characterization of *Lactobacillus* isolates from traditional Manchego cheese for potential use as adjunct starter cultures. Food Control 20:1092–1098

Nieto-Arribas P, Sesena S, Poveda JM, Palop L, Cabezas L (2010) Genotypic and technological characterization of *Leuconostoc* isolates to be used as adjunct starters in Manchego cheese manufacture. Food Microbiol 27:85–93

Rademaker JLW, Peinhopf M, Rijnen L, Bockelmann W, Noordman WH (2005) The surface microflora dynamics of bacterial smear-ripened Tilsit cheese determined by T-RFLP DNA population fingerprint analysis. Int Dairy J 15:785–794

Rattray FP, Fox PF (1999) Aspects of enzymology and biochemical properties of *Brevibacterium linens* relevant to cheese ripening: a review. J Dairy Sci 82:891–909

Ummadi M, Weimer BC (2001) Tryptophan catabolism in *Brevibacterium linens* as a potential cheese flavor adjunct. J Dairy Sci 84:1773–1782

Valdes-Stauber N, Scherer S, Seiler H (1997) Identification of yeasts and coryneform bacteria from the surface microflora of brick cheese. Int J Food Microbiol 34:115–129

Vauterin L, Vauterin P (1992) Computer-aided objective comparison of electrophoretic patterns for grouping and identification of microorganisms. Eur Microbiol 1:37–41

Weimer B, Dias B, Ummadi M, Broadbent J, Brennand C, Jaegi J, Johnson MJ, Milani F, Steele J, Sisson DV (1997) Influence of NaCl and pH on intracellular enzymes that influence Cheddar cheese ripening. Lait 77:383–398

Zemanovic J, Valik L, Gorner F (1992) Influence of water activity on growth and production of *Brevibacterium linens* proteinases. Bull PV 31(4):249–256