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# STUDY OF PROTEOLYSIS IN MINIATURE TOMA PIEMONTESE CHEESE MADE USING WILD BACTERIA

STUDIO DELLA PROTEOLISI IN FORMAGGI MINIATURIZZATI  
DI TOMA PIEMONTESE PRODOTTI USANDO BATTERI AUTOCTONI

M. BERTOLINO\*, G. ZEPPA, V. GERBI and P.L.H. McSWEENEY<sup>1</sup>

Dipartimento di Valorizzazione e Protezione delle Risorse Agroforestali,  
Settore di Microbiologia Agraria e Tecnologie Alimentari, Facoltà di Agraria,  
Università degli Studi di Torino, Via Leonardo da Vinci 44,  
10095 Grugliasco (TO), Italy

<sup>1</sup>Department of Food and Nutritional Science, University College, Cork, Ireland

\*Corresponding author: Tel. +39 011 6708705, Fax +39 011 6708549,  
email: marta.bertolino@unito.it

## ABSTRACT

The effects of 35 strains of indigenous bacteria on proteolysis in model Toma Piemontese cheese were studied at 30 and 60 days of ripening. Proteolysis was assessed by urea-polyacrylamide gel electrophoresis (PAGE) of the pH 4.6-insoluble fractions of cheese, by reversed-phase high performance liquid chromatography (RP-HPLC) of the pH 4.6-soluble fractions at 30 and 60 days of ripening and by free amino acid (FAA) analysis at 60 days of ripening. Urea-PAGE showed that  $\alpha_{s1}$ -casein was hydrolysed more than  $\beta$ -casein. The use of

## RIASSUNTO

In questa ricerca è stato studiato l'effetto prodotto dall'utilizzo di 35 ceppi di batteri lattici autoctoni sulla proteolisi in formaggi modello di Toma Piemontese. La proteolisi è stata valutata a 30 e a 60 giorni attraverso l'elettroforesi su gel di urea-poliacrilamide (Urea-PAGE) della frazione insolubile a pH 4,6 e la cromatografia liquida ad alte prestazioni in fase inversa (RP-HPLC) della frazione solubile a pH 4,6 nonché attraverso l'analisi degli amino acidi liberi (FAA) sui formaggi a 60 giorni di stagionatura. L'urea-PAGE ha mostrato

- Key words: indigenous strains, proteolysis, Toma Piemontese cheese -

single strains markedly influenced the peptide profiles obtained by RP-HPLC of the cheeses. Significant differences in the levels of FAAs were also observed among the cheeses. The results of this study highlighted marked differences in the acidification and proteolytic activities of these bacterial strains.

come l' $\alpha_{s1}$ -caseina sia stata idrolizzata di più rispetto alla  $\beta$ -caseina, mentre la RP-HPLC ha evidenziato l'effetto dei diversi ceppi batterici utilizzati sui profili peptidici. Significative differenze sono state rilevate inoltre nel livello di FAA e ciò conferma la differente capacità proteolitica dei ceppi saggiati.

## INTRODUCTION

In ripening, cheese is subjected to three major categories of biochemical events: glycolysis, lipolysis and proteolysis (FOX, 1989). However, proteolysis is the most important process that occurs during ripening because it contributes to texture through the hydrolysis of the protein matrix and to flavour and off-flavour formation as a direct consequence of the production of peptides and free amino acids and indirectly by the liberation of amino acids that can be subjected to other changes including decarboxylation, deamination or desulphuration to produce other flavour compounds (SOUSA *et al.*, 2001).

Proteolysis in cheese is catalysed by proteinases and peptidases derived from six sources: coagulant (chymosin, pepsin, fungal acid proteinase or plant acid proteinases); milk (plasmin, cathepsin D and other somatic cell proteinases); starter lactic acid bacteria; adventitious non-starter lactic acid bacteria; addition of secondary starters in some cheese varieties (*Penicillium roqueforti*, *Penicillium camemberti*, *Geotrichum candidum*) and exogenous proteinases and/or peptidases which may be added to accelerate ripening or to accentuate flavour of the cheese (FOX and McSWEENEY, 1996; UPADHYAY *et al.*, 2004). Proteolysis in cheese can be divided into two phases: primary proteolysis which is the initial hydrolysis of caseins by the coagulant and the milk proteinases to produce the

large and intermediate-sized peptides that are subsequently degraded during secondary proteolysis. During primary proteolysis, chymosin, the principal enzyme in rennets used in cheesemaking, acts on  $\alpha_{s1}$ -casein at Phe<sub>23</sub>-Phe<sub>24</sub>. Plasmin, the indigenous alkaline milk proteinase, cleaves the  $\beta$ -casein to give  $\gamma$ -caseins ( $\beta$ -CN f29-209,  $\beta$ -CN f106-209 and  $\beta$ -CN f108-209). The products of the hydrolysis of  $\alpha_{s1}$ - and  $\beta$ -caseins can be detected by polyacrylamide gel electrophoresis (urea-PAGE) (GRAPPIN *et al.*, 1985). During secondary proteolysis enzymes derived from the starter and non-starter flora of cheese act on the products of primary proteolysis and produce small peptides and free amino acids. These products can be extracted as the water-soluble fractions and analysed by reversed-phase high performance liquid chromatography (RP-HPLC) (RANK *et al.*, 1985; ARDÖ and GRIPON, 1991; McSWEENEY and FOX, 1997).

Lactic acid bacteria are very important for dairy production; they have a very complex proteolytic system composed of a proteinase and a wide range of peptidases that are responsible for the formation of small peptides and amino acids in cheese. The proteolytic systems of *Lactococcus* spp. and of *Lactobacillus* spp. have been studied in great detail because of their economic value as starter cultures in dairy fermentations (NORAINI and ELMER, 1990; BOCKELMANN, 1995).

Bacteria which take part in the differ-

ent biochemical processes that occur in cheese during ripening come from starters added during cheesemaking or from the environment but the former are the most important as they are deliberately added and increase to high numbers.

In many European countries, cheeses (e.g. Beaufort, Comté, Grana Padano and Parmigiano Reggiano) are traditionally made using natural cultures obtained by incubating milk or whey from the previous day's production under defined conditions. The inoculum is characterised by a well-balanced flora of mesophilic and thermophilic lactic acid bacteria. Nevertheless, natural cultures are characterised by a high variability in strain composition over time and require a long time to be prepared. It is now normal practice to make cheese using commercial starter cultures that are easy to use; the cheeses that are produced are standardised but with some organoleptic impoverishment (GRAPPIN and BEUVIER, 1997; MENÉNDEZ *et al.*, 2004). As a consequence, interest has been shown in the genotypic and technological characterisation of wild isolates from artisanal cheeses because new strains could be selected and used as defined-strain cultures to give cheeses with technological and organoleptic characteristics that are more similar to traditional cheese (COGAN *et al.*, 1997; SHAKEEL-UR-REHMAN *et al.*, 1999; BERESFORD *et al.*, 2001; GONZALES *et al.*, 2003; POVEDA *et al.*, 2003; HAYALOGU *et al.*, 2004; DI CAGNO *et al.*, 2006).

In Italy, these autochthonous starter cultures are currently being used to produce many Protected Denomination of Origin (PDO) cheeses such as Asiago, Bitto, Fontina and Pecorino Toscano but they have not been used for Toma Piemontese which is the most important cheese from the northwestern Alps.

Toma Piemontese PDO cheese was traditionally made in mountain farms using raw milk. However, over the last decade, it has been manufactured on a large

scale in small and large dairy plants using pasteurized milk with the addition of industrial freeze-dried starters that generally contain mesophilic lactic acid bacteria. Toma Piemontese is a cylindrical, semi-hard, semi-cooked cheese. It is produced in two sizes, large (6-8 kg, 24-34 cm in diameter) and small (1.8-5.9 kg, 15-25 cm in diameter). The milk is cooled to 37°C and the rennet is added at a concentration of 0.15-0.20 mL × L<sup>-1</sup>. The coagulation time is established visually by the producer. The curd is then cut into 5-10 mm pieces, placed in a cloth, pressed, drained for 24 h and then salted. Cheese is ripened in a cold room or caverns at 6-10°C and 85% humidity for 30-60 days.

To date the proteolysis in Toma Piemontese cheese has not been examined nor has the use of single artisanal strains. The objective of the present study was to evaluate the effect of thirty-five strains of LAB isolated from artisanal Toma Piemontese cheeses on proteolysis in model cheeses after 30 or 60 days of ripening.

## MATERIALS AND METHODS

### Cultures

*Lactococcus lactis* subsp. *lactis* (A 1.1, A 1.4, A 1.6, A 1.13, A 1.18, A 1.20, A 2.1, A 2.5, TA 1.2, TA 2.1, TA 2.4, TA 2.5, TB 2.1, TB 2.2, TB 2.4, TB 2.6, TB 2.7, TB 2.8, TC 1, TC 6 and TC 8), *Lactococcus lactis* subsp. *cremoris* (A 1.8 and A 2.4), *Lactobacillus paracasei* (TO 1.1 and TA 1.4), *Streptococcus thermophilus* (B 1.18, A 2.3 and TA 1.1), *Streptococcus macedonicus* (TA 3.1, TA 3.4, TA 3.5, TB 1.4 and TB 1.9), *Lactobacillus fermentum* (TA 2.2) and *Lactobacillus casei* subsp. *rhamnosus* (TA 3.3) were used separately as starter cultures. These organisms were isolated from artisanal Toma Piemontese cheeses and characterised as reported by FORTINA *et al.* (2003) and were obtained

from the culture collection of the Department of Exploitation and Protection of the Agricultural and Forestry Resources, Agricultural Microbiology and Food Technologies Section, Agricultural Faculty, University of Turin. Before use, each strain was cultivated in M17 broth (Merck, Darmstadt, Germany) for two transfers at 37°C for 24 h (1% inoculum). For starter propagation, the broth cultures were grown in reconstituted skim milk (10 g × 100 mL<sup>-1</sup> autoclaved for 10 min at 115°C) with an inoculum of 1%. Before their use as inoculum for cheesemaking, a microscopic count was done on the cultures of each strain in order to standardise the concentration of inoculum added to the milk at 10<sup>7</sup> cfu mL<sup>-1</sup>.

### Cheese manufacture

Model cheeses were made using the method described by SHAKEEL-UR-REHMAN *et al.* (1998) but modified to produce cheeses that were more similar to Toma Piemontese cheese.

No control cheese was included in this study because the only possible control cheeses could have been cheese made from raw milk, a starter-free cheese or the use of a commercial starter but none of these types of cheeses would have been a good control. In the first case raw milk would have had a very heterogeneous microflora which would not have been possible to replicate day by day. Many studies have been conducted on starter-free cheese, and the results have shown that the differences between starter-free and starter cheese is much greater than any possible strain-to-strain differences. The use of a commercial starter as a control would have simply been another strain to be tested. Moreover all the other parameters in cheesemaking were the same for each strain so that the differences in the final cheeses were determined by the different strains used.

For each strain, six model cheeses were made. Raw milk was pasteurised in

beakers at 63°C for 30 min. Pasteurised milk was put into plastic centrifuge bottles (200 mL) cooled to 37°C and maintained at this temperature in a water bath. A starter inoculum of 2% was added to milk (corresponding to 10<sup>7</sup> cfu mL<sup>-1</sup>) which was ripened for 15 min. CaCl<sub>2</sub> (132 µL of a 1 mol × L<sup>-1</sup> solution/200mL milk) was then added and held for 15 min. Rennet (Maxiren-180, DSM, Seclin Cedex, France) was added (43.5 µL/200 mL) and the milk was held for 45 min to obtain a firm coagulum. After the coagulum was cut, it was held for 2 min and then stirred slowly for 10 min using a glass rod. The temperature was then increased to 41°C over 30 min. Curd and whey were then centrifuged at room temperature for 60 min at 1700 g in an MSE 'MAJOR' centrifuge (MSE Scientific Instruments, Crawley, Sussex, England). The whey was drained and the curds were covered with 3 mL of sterile water and then kept in the centrifuge bottles at 30°C for 18 hours. The water was drained and the cheeses were inverted in the bottles and then recentrifuged at 1700 g for 20 min. The cheeses were then brine salted (20% NaCl, 0.05% CaCl<sub>2</sub> × 2H<sub>2</sub>O) for 30 min at room temperature. After salting, cheeses were removed from the bottles wiped with tissue paper and vacuum packed. They were ripened at 8°C for 30 and 60 days. At the end of the ripening period the weight of the miniature cheeses ranged from 20 to 22 g. Cheeses were sampled for compositional analysis on day 30 and for proteolysis on days 30 and 60.

### Composition analysis

Cheeses were analysed at 30 days for moisture by the oven drying method at 102°C (IDF, 1982) and for salt by titration with AgNO<sub>3</sub> according to the method of BRADLEY *et al.* (1993). The pH was determined at 30 and 60 days by placing a penetration electrode in contact with the cheese mass.

## Assessment of proteolysis

After ripening for 30 and 60 days, pH 4.6-insoluble and -soluble fractions were prepared by a slight modification of the method of KUCHROO and FOX (1982) as described by HAYALOGLU *et al.* (2004). Instead of using 20 g of cheese and 40 mL of water for the extraction, 15 g of cheese and 30 mL of water were used.

Urea-polyacrylamide gel electrophoresis (Urea-PAGE) [12% T (total monomer), 4% C (crossing-linking agent as percentage of total monomer), pH 8.9] of the pH 4.6-insoluble fractions of the cheeses was performed using a Protean II xi vertical slab-gel unit (Bio-Rad Laboratories Ltd., Watford, UK) according to the method of ANDREWS (1983) with the modifications of SHALABI and FOX (1987). The gels were stained directly with Coomassie Brilliant Blue G-250 by the method of BLAKESLEY and BOEZI (1977) and destained using distilled water. After destaining, gel slabs were digitised with a scanner (Scanjet 6300C, Hewlett Packard, Houston, USA). Scans of the electrophoretograms were used to quantify bands using densitometric software (Image Master TotalLab 1D Gel analysis v 1.11 software, Nonlinear Dynamics Ltd, Newcastle-upon-Tyne, UK). The caseins and peptides were quantitatively determined by integration of peak volumes using a densitometer.

Peptides of the pH 4.6-soluble fraction of the cheese were determined by RP-HPLC using the method described by HAYALOGLU *et al.* (2004).

For the free amino acid (FAA) analysis, the pH 4.6-soluble fractions of the cheeses were deproteinised by mixing them with an equal volumes of 24% (w/v) trichloroacetic acid (TCA). The solutions were allowed to stand for 10 min before centrifugation at 14400 *g* for 10 min (MSE, Microcentaur, DJB Labcare Ltd, Newport Pagnell Buckinghamshire, UK). The supernatant was removed and diluted with 0.2 M sodium citrate buffer

(pH 2.2) to give approximately 250 nmol of each amino acid residue. The samples were then diluted 1:2 with the internal standard norleucine to give an approximate final concentration of 125 nmol of each amino acid residue in 1 mL of solution. Samples were then analysed using a Jeol JLC-500/V amino acid analyser (Jeol Ltd., Garden city, Herts, UK) fitted with a Jeol Na<sup>+</sup> high performance cation-exchange column. The individual free amino acids were separated by ion-exchange chromatography with post-column ninhydrin derivatization and visible colorimetric detection at 570 nm. Results were obtained using a Aminotag data handling system (J & C Joel Ltd, Halifax, UK).

## Statistical analysis

Two cheeses for each strain were used for each analysis. The data used for the statistical analysis were the means of these two replicates.

Data from urea-PAGE gels of the pH 4.6-insoluble fractions were analysed using multivariate statistical analysis. Similar bands in the urea-PAGE gels were recognised visually as described by McSWEENEY *et al.* (1994) and the peak volume of corresponding bands were used as data for cluster analysis (CA). Data from RP-HPLC of the pH 4.6-soluble fractions were analysed using CA to evaluate the effect of the single-strain starters. Data for the statistical analysis of the RP-HPLC chromatograms were obtained by visually recognising similar peaks in the chromatograms and using peak heights as variables as described in PRIPP *et al.* (1999). The peak heights were obtained by converting the corresponding chromatogram to an ASCII file. CA was performed by using Euclidean distance without standardising the variables. Statistical analyses were performed using Statistica for Windows (StatSoft for Windows Ver. 7, Tulsa, OK, USA).



## RESULTS AND DISCUSSION

### Compositional analysis

After 30 days of ripening, the range of compositional parameter values of all the cheeses analysed were: 56-57% moisture, 1.5-1.6% salt and pH 5.11. These values are close to those of Toma Piemontese cheese (moisture 46-54%, salt 0.8-3.5 and pH 5.00-5.52) (AMBROSOLI *et al.*, 1998).

### Urea-PAGE

Urea-PAGE electrophoretograms of the pH 4.6-insoluble fractions of cheese samples after 30 days of ripening are shown in Fig. 1. The peptide profiles obtained by the urea-PAGE were the result of the direct effects of the rennet and indigenous milk enzymes and the effects of pH on the activities caused by some indi-

rect effects of the strains used as starter. The lactic acid bacteria used as starters had different acidification kinetics that resulted in different final pH values in the cheeses in which they were used as starters (Table 1). These differences in pH affected the breakdown of the caseins. The patterns of proteolysis were almost identical in all the cheeses. It can be seen that neither  $\beta$ -casein nor  $\alpha_{s1}$ -casein were degraded very extensively. The cheese made with the *Lactobacillus paracasei* TA1.4 and *Streptococcus macedonicus* TA 3.4 strains showed the highest degradation of  $\beta$ -casein followed by those made with *Lactococcus lactis* subsp. *lactis* TA 2.5 and *Streptococcus macedonicus* TA 3.5. These cheeses had a pH of 5.30, 5.29, 5.25 and 5.24, respectively, which would have favoured plasmin action. With respect to  $\gamma$ -caseins, the polypeptides produced by the action of plasmin on  $\beta$ -casein,  $\gamma_3$ -casein

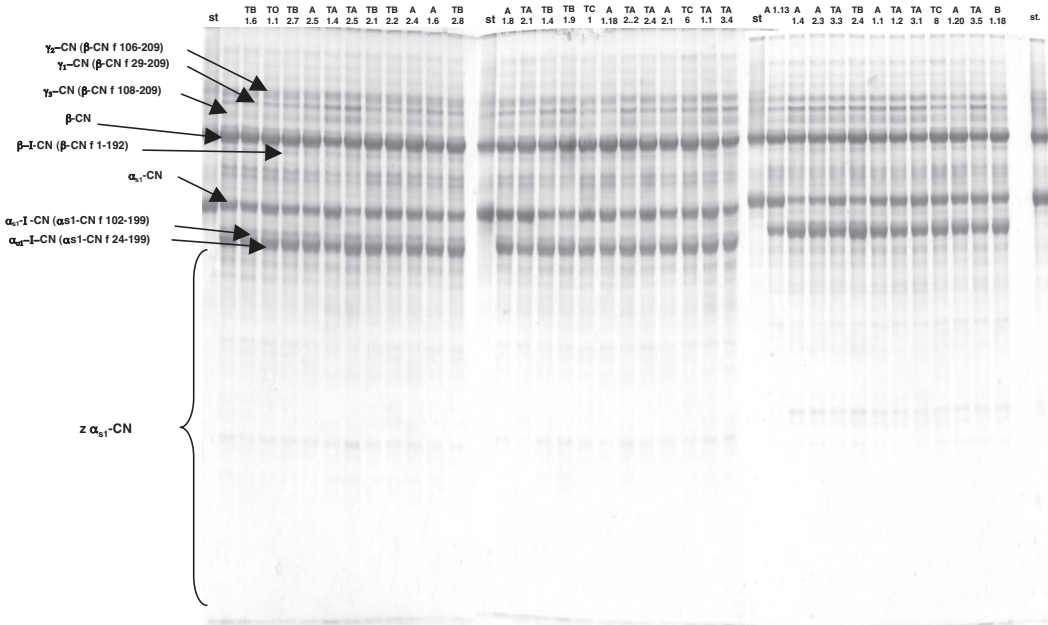


Fig. 1 - Urea-polyacrylamide gel electrophoretograms of miniature Toma Piemontese cheeses made by using different single strains of indigenous bacteria after 30 days of ripening. st = standard Na-caseinate.

Table 1 - pH value of the cheeses made by using single starter strains of lactic acid bacteria after 30 and 60 days of ripening.

Strains	A 1.13	A 1.18	A 1.20	B 1.18	A 1.8	TO 1.1	TA 2.5	TB 2.4	A 2.1	TA 1.2	TB 1.4	TB 1.9
30 days ripening	5.16	5.11	5.24	5.02	5.05	4.90	5.25	5.19	5.12	5.14	5.09	5.09
60 days ripening	5.08	5.04	5.08	4.91	5.04	4.90	5.04	5.06	5.11	5.03	5.08	4.86
Strains	TC 6	TA 3.4	TA 2.2	TA 3.3	A 1.6	A 1.1	A 1.4	A 2.5	TA 2.1	TA 2.4	TB 2.1	TB 2.2
30 days ripening	5.13	5.29	5.11	5.19	5.04	5.20	5.16	5.01	5.06	5.11	5.02	5.02
60 days ripening	5.06	5.10	5.10	5.25	5.03	5.15	5.16	5.00	5.04	5.09	5.01	5.00
Strains	TB 2.6	TB 2.7	TB 2.8	TC 1	TC8	A 2.3	A 2.4	TA 1.1	TA 3.1	TA 3.5	TA 1.4	
30 days ripening	4.86	4.93	5.04	5.09	5.23	5.16	5.03	5.14	5.25	5.24	5.30	
60 days ripening	4.86	4.90	5.03	5.09	5.10	5.14	5.01	5.12	5.20	5.22	5.17	

was present at low concentrations in all the cheeses except for the one made using the *Lactococcus lactis* subsp. *lactis* TA 1.2 strain.  $\gamma_2$ -Casein was present in the highest concentrations in 26 cheeses of the 35 cheeses analysed, most of which were made using *Lactococcus lactis* subsp. *lactis*.  $\gamma_1$ -Casein was present in the highest concentrations in 8 cheeses which were produced by using the *Lactococcus lactis* subsp. *lactis* A 1.4, A 2.3, TB 2.4, A 1.1, *Streptococcus macedonicus* TA 3.1, TA 3.5, B 1.18 and *Lactobacillus casei* subsp. *rhamnosus* TA 3.3 strains. A faint band that corresponded to  $\beta$ -I-casein ( $\beta$ -CN f 1-192), derived from the action of chymosin on  $\beta$ -casein, was present in the electrophoretograms of all the cheeses.

The cheeses made using *Lactococcus lactis* subsp. *lactis* TA 2.5, A 1.4, TB 2.4 and *Streptococcus thermophilus* A 2.3 strains were characterised by the highest degradation of  $\alpha_{s1}$ -casein. In all the electrophoretograms, the band corresponding to the  $\alpha_{s1}$ -I-casein ( $\alpha_{s1}$ -CN f 24-199), which is the product of rennet action on  $\alpha_{s1}$ -casein, was present. The highest concentrations of this peptide were obtained with *Lactococcus lactis* subsp. *lactis* TA 2.5, TB 2.4, *Lactococcus lactis* subsp. *cremoris* A 1.8 and *Streptococcus macedonicus* TB 1.4 strains. In all the cheeses, other peptides (marked as z  $\alpha_{s1}$ -CN on the electrophoretograms shown in

Fig. 1) were present with electrophoretic mobilities that were faster than  $\alpha_{s1}$ -I-casein; they may have been derived through the action of rennet and indigenous milk proteinases.

Urea-PAGE electrophoretograms of the pH 4.6-insoluble fractions of cheese samples after 60 days of ripening are shown in Fig. 2. After 2 months of ripening, the  $\beta$ -casein was less hydrolysed than  $\alpha_{s1}$ -casein. Many authors have discussed the fact that  $\beta$ -casein is more resistant to hydrolysis than  $\alpha_{s1}$ -casein during ripening (FOX *et al.*, 1993; SARANTINOPOULOS *et al.*, 2002; HAYALOGLU *et al.*, 2004; FALLICO *et al.*, 2006).  $\gamma_1$ -Casein was present in the highest concentration among the  $\gamma$ -caseins in all cheeses except those that were made using *Lactococcus lactis* subsp. *lactis* TB 2.8 and TC 1 in which  $\gamma_3$ -casein and  $\gamma_2$ -casein were present in the highest concentrations, respectively. Moreover, all the cheeses were characterised by a faint band corresponding to  $\beta$ -I-casein ( $\beta$ -CN f 1-192). With respect to  $\alpha_{s1}$ -casein, all the 60-day-old cheeses were characterised by a greater amount of hydrolysis than the 30-day-old cheeses. The strains that degraded this casein the most were *Lactococcus lactis* subsp. *lactis* A 2.5, TA 2.5, TB 2.1, TB 2.4 and *Streptococcus thermophilus* A 2.3. The cheeses that contained the highest concentrations of  $\alpha_{s1}$ -I-casein ( $\alpha_{s1}$ -CN f 24-199) were those



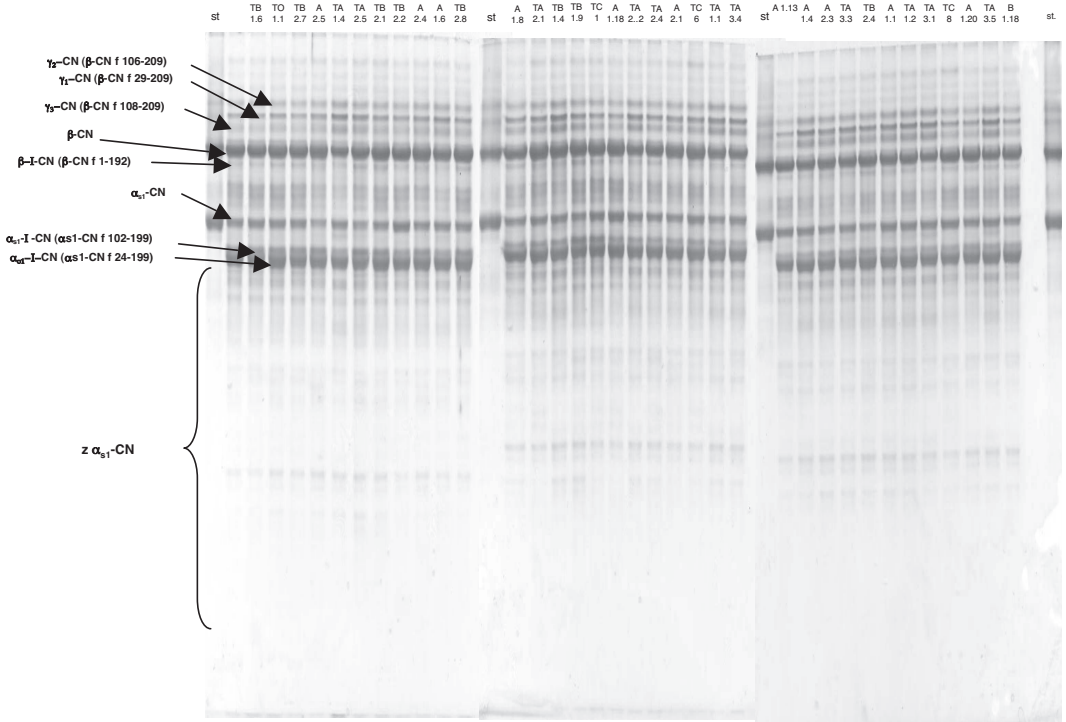


Fig. 2 - Urea-polyacrylamide gel electrophoretograms of miniature Toma Piemontese cheeses made by using different single strains of indigenous bacteria after 60 days of ripening. st = standard Na-caseinate.

that were made by using *Lactobacillus paracasei* TO 1.1, *Lactobacillus fermentum* TA 2.2, *Streptococcus thermophilus* B 1.18, *Lactococcus lactis* subsp. *cremoris* and *Lactococcus lactis* subsp. *lactis* TB 2.6 and A 1.20. In all the cheeses, high levels of other peptides were present (marked as  $z \alpha_{s1}$ -CN on the electrophoretograms shown in Fig. 2) that had electrophoretic mobilities that were faster than  $\alpha_{s1}$ -I-casein. These may have been derived from the action of rennet and indigenous milk proteinases.

The results of the CA of the peak volumes of the bands on the electrophoretograms of the cheeses after 60 days of ripening divided the strains into two groups as shown in the dendrogram in Fig. 3. The first group was made up of 18 *Lactococcus lactis* subsp. *lactis*, 2 *Lactococcus lactis* subsp. *cremoris*, 2 *Strepto-*

*coccus macedonicus*, 1 *Lactobacillus paracasei* and 1 *Streptococcus thermophilus* strain. The second group was made up of 3 *Lactococcus lactis* subsp. *lactis*, 3 *Streptococcus macedonicus*, 2 *Streptococcus thermophilus*, 1 *Lactobacillus fermentum*, 1 *Lactobacillus paracasei* and 1 *Lactobacillus casei* subsp. *rhamnosus* strain. The cheeses made using strains belonging to the first group were characterised by a greater degradation of the  $\alpha_{s1}$ -casein compared to cheeses made using strains from the second group that were characterised by a greater degradation of  $\beta$ -casein.

#### RP-HPLC

The results of a multivariate statistical analysis of the pH 4.6-soluble fractions of the 30-day-old cheeses, based

on the height of 35 peaks, divided the strains into two groups as shown in the dendrogram in Fig. 4. The first group was composed of 5 *Streptococcus macedonicus* strains, 4 *Lactococcus lactis* subsp. *lactis*, 2 *Streptococcus thermophilus*, 1 *Lactobacillus paracasei*, 1 *Lactobacillus fermentum* and 1 *Lactobacillus casei* subsp. *rhamnosus* strain. The second group was composed of 17 *Lactococcus lactis* subsp. *lactis* strains, 2 *Lactococcus lactis* subsp. *cremoris*, 1 *Streptococcus thermophilus* and 1 *Lactobacillus paracasei* strain. RP-HPLC chromatograms of the pH 4.6-soluble fractions from the 30-day-old miniature Toma Piemontese cheeses made using *Lactococcus lactis* subsp. *lactis* A 1.1

(belonging to the first group) and *Lactococcus lactis* subsp. *lactis* A 1.6 (belonging to the second group) as starter are shown in Fig. 5. The peaks that were recognised and used as variables in the multivariate statistical analysis are indicated. The two groups were characterised by large differences in the heights of peaks 1,6,16,25,26 and 27. The highest peaks of the strains belonging to the first group were peaks 3,4,5,8,10-13,15-18 and 20-29. Most of these peaks were eluted in the chromatogram at retention times from 40 to 55 min and hence were principally large hydrophilic peptides (GONZÁLEZ DEL LLANO *et al.*, 1995; PAVIA *et al.*, 2000). The second group, principally made up of *Lactococcus* spp.,

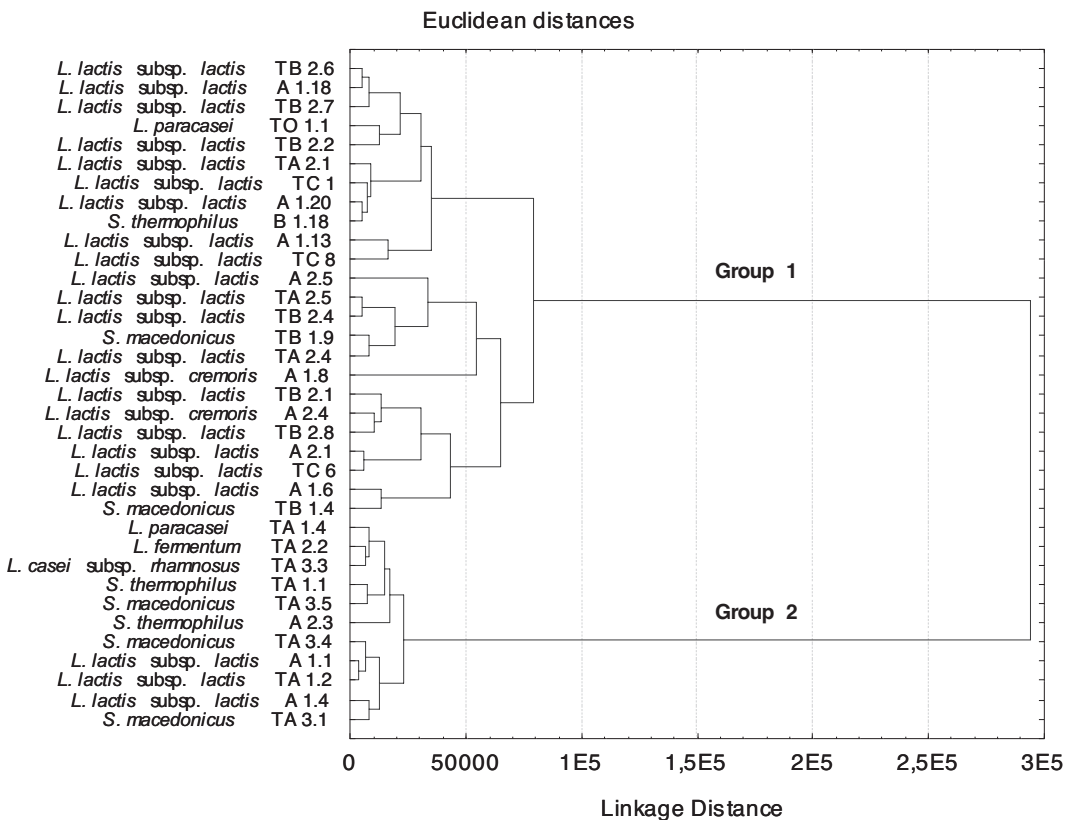


Fig. 3 - Dendrogram from cluster analysis of data obtained from urea-polyacrylamide gel electrophoretograms of pH 4.6-insoluble fractions of the cheeses made by using different single strains of indigenous bacteria after 60 days of ripening.

was characterised by the highest values for peaks 1,2,6,7,9,14,19, and 30-35. These peaks were eluted in the first and last parts of the chromatogram at retention times that were lower than 35 min or higher than 55 min and hence were comprised principally of amino acids and hydrophilic peptides and hydrophobic peptides, respectively (GONZÁLEZ DEL LLANO *et al.*, 1995; POVEDA *et al.*, 2003; HAYALOGLU *et al.*, 2004).

The CA of the pH 4.6-soluble fractions of the 60-day-old cheeses, based on the heights of 48 peaks, grouped the strains in the same way as for 30 day-old cheeses except for the *Lactococcus lactis* subsp. *lactis* TB 2.4, *Lactococcus lactis* subsp. *lactis* TA 2.5 and *Streptococcus macedonicus* TB 1.9 strains as shown in

the dendrogram in Fig. 6. The RP-HPLC chromatograms of the pH 4.6-soluble fractions from the 60-day-old miniature Toma Piemontese cheeses made using *Lactococcus lactis* subsp. *lactis* A 1.1 (belonging to the first group) and *Lactococcus lactis* subsp. *lactis* A1.6 (belonging to the second group) as starter are shown in Fig. 7. The peaks that were recognised and used as variables in the multivariate statistical analysis are indicated. The two groups were characterised by a notable difference in the heights of peaks 1, 4, 5, 10, 11, 13, 16, 18, 20, 21, 22, 26, 27,29 and 34-40. The strains belonging to the first group were clustered because of the high values of peaks 27 and 37-39 relative to the strains belonging to the second group. Moreover, the

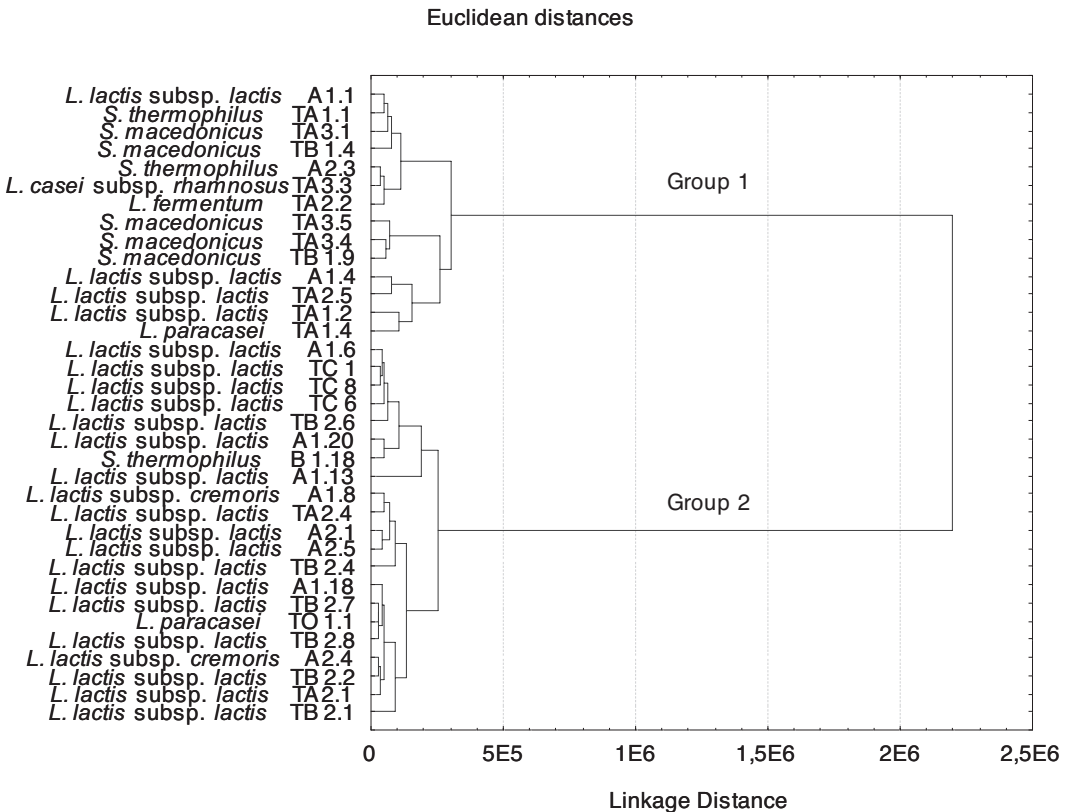


Fig. 4 - Dendrogram from cluster analysis of data obtained from RP-HPLC pH 4.6-soluble fractions of the cheeses made by using different single strains of indigenous bacteria after 30 days of ripening.

strains that formed the second group were clustered because of the high values of peaks 1,4,5 and 13.

### Individual free amino acids

Proteolysis in the cheeses was also monitored by determining the levels of individual free amino acids (FAAs)

as shown in Fig. 8. The principal FAAs present in 60-day-old cheeses were Leu, Glu, Phe, His and Lys. Cheeses made using the *Lactococcus lactis* subsp. *lactis* strains had the highest concentrations of Leu, Phe, His, Lys, Cys, Met, Ser, Met, Thr and Ile. The cheeses made using *Lactococcus lactis* subsp. *cremoris* strains had the highest concentration of Val

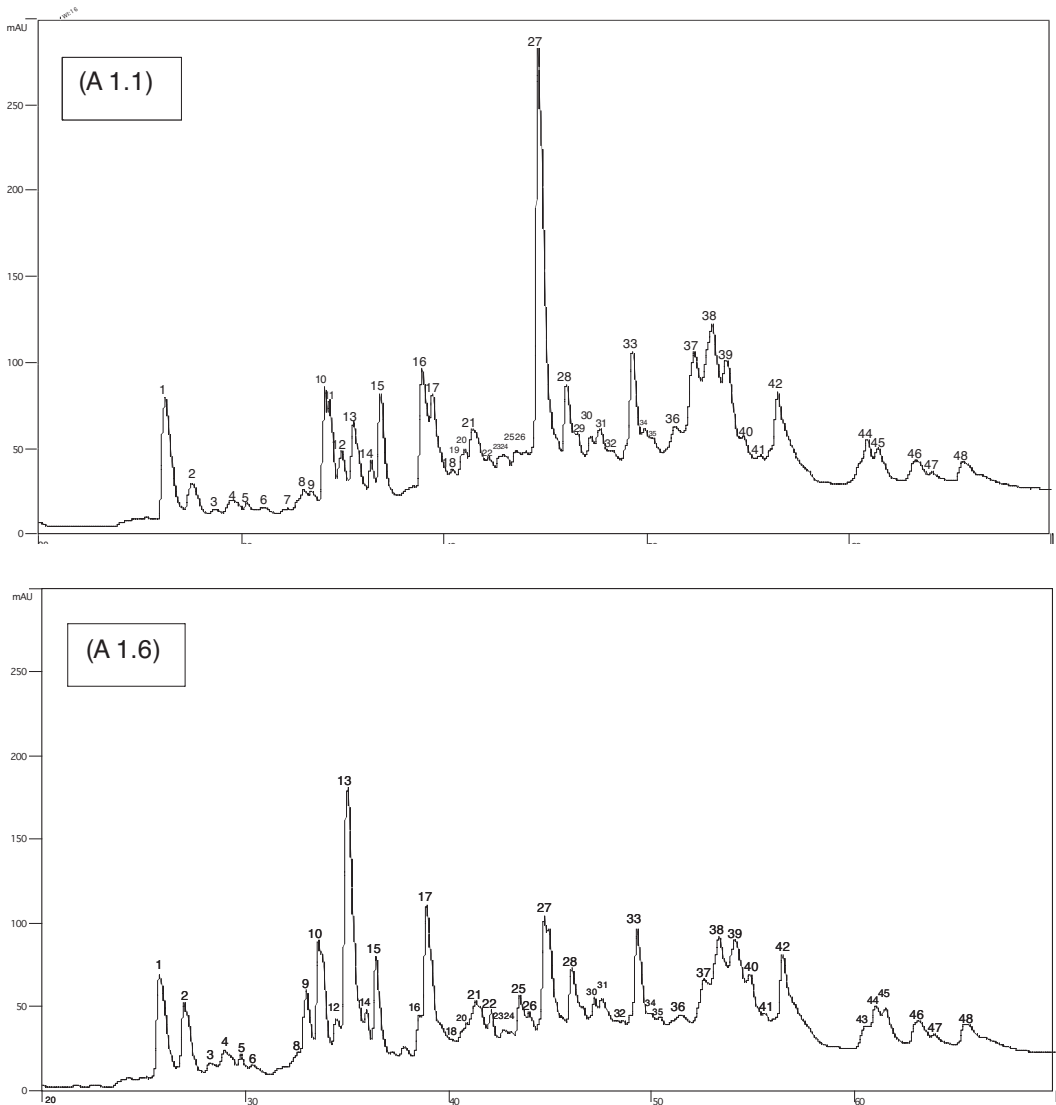


Fig. 5 - RP-HPLC profiles of pH 4.6-soluble fractions of miniature Toma Piemontese cheese made by using the strains *Lactococcus lactis* subsp. *lactis* A 1.1 and A 1.6 after 30 days of ripening.

and Gly and the lowest concentrations of Arg. The cheeses made using *Streptococcus macedonicus* strains had the lowest concentrations of Lys while those made using *Streptococcus thermophilus* strains had the lowest concentrations of Gly. The cheeses made using *Lactobacillus paracasei* strains were characterised by the lowest concentrations of Thr while that made using *Lactobacillus casei* subsp. *rhamnosus* strain was characterised by the highest concentrations of Glu, Asp and Arg and the lowest concentrations of Pro and Met. The cheese made using *Lactobacillus fermentum* strain had the lowest concentrations of Arg, Thr, Ser, Cys, Met, Ile, Leu, Tyr, Try, Phe, His, Arg and Pro. The results

of the CA of the FAA data showed that the strains were divided into two groups as shown in the dendrogram in Fig. 9. The first group was made up of 16 *Lactococcus lactis* subsp. *lactis* strains and 1 *Lactococcus lactis* subsp. *cremoris* strain. The second group was made up of 6 *Streptococcus macedonicus* strains, 5 *Lactococcus lactis* subsp. *lactis*, 3 *Streptococcus thermophilus*, 1 *Lactococcus lactis* subsp. *cremoris*, 1 *Lactobacillus casei* subsp. *rhamnosus* and 1 *Lactobacillus fermentum* strain. The two groups were characterised by marked differences in the levels of Leu, Glu, Phe, His, Val, Lys and Pro. The cheeses made using the strains belonged to the second group were characterised by the high-

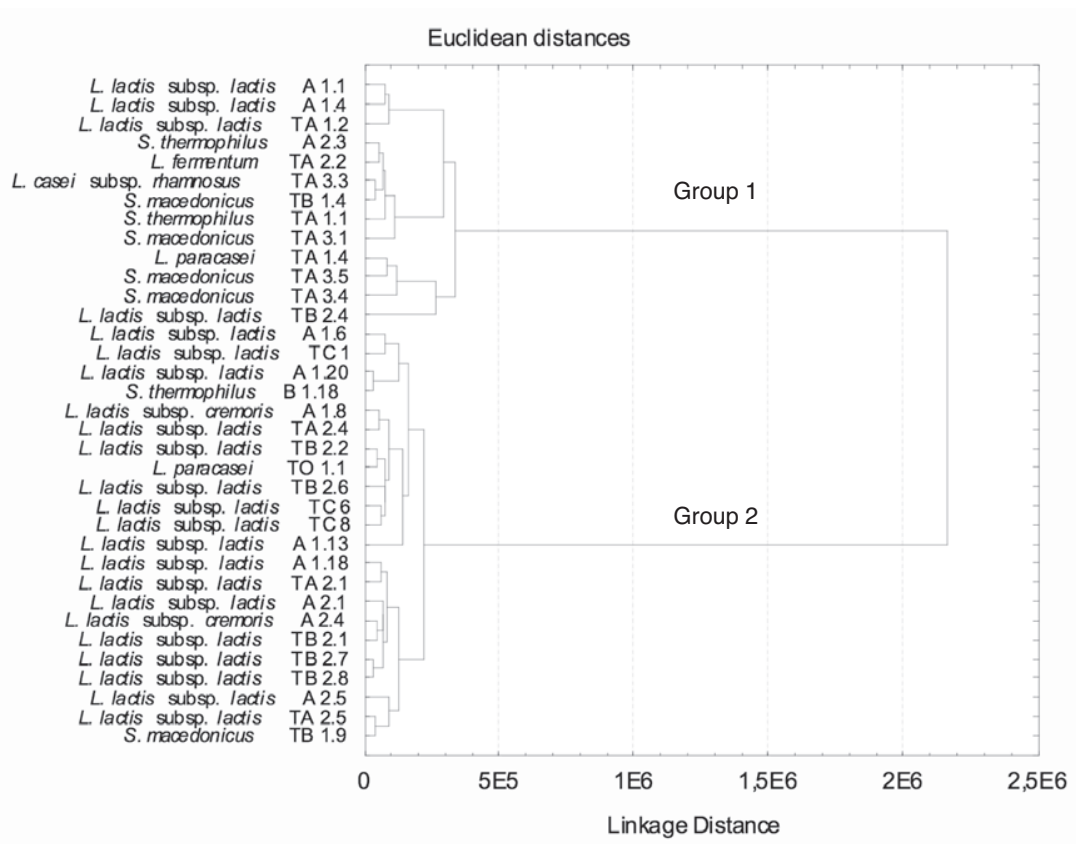


Fig. 6 - Dendrogram from cluster analysis of data obtained from RP-HPLC pH 4.6-soluble fractions of the cheeses made by using different single strains of indigenous bacteria after 60 days of ripening.

est concentrations of all the FAAs except Ala. Starter peptidases are responsible for the production of amino acids (McSWEENEY *et al.*, 1993; LANE and FOX, 1996; CORSETTI *et al.*, 1998). The levels of free amino acids liberated varied due to differences in the enzyme systems of the single strains and the factors that influence their level of autolysis during cheese ripening (WILKINSON *et al.*, 1994a,b; KUNJI *et al.*, 1996; CHRISTENSEN *et al.*, 1999; SAVIJOKI *et al.*, 2006). Other researchers have stud-

ied the effect of single strains of lactic acid bacteria on the production of FAAs. O'DONOVAN *et al.* (1996) studied the autolytic properties of three *Lactococcus* strains during cheese ripening. Their research showed that the concentration of FAAs increased in all the cheeses during ripening and that Glu, Leu, and Lys were the major FAAs present at all of the ripening stages. HYNES *et al.* (2001) studied the effect of adding *Lactobacillus plantarum* to different strains of starter bacteria in the production of

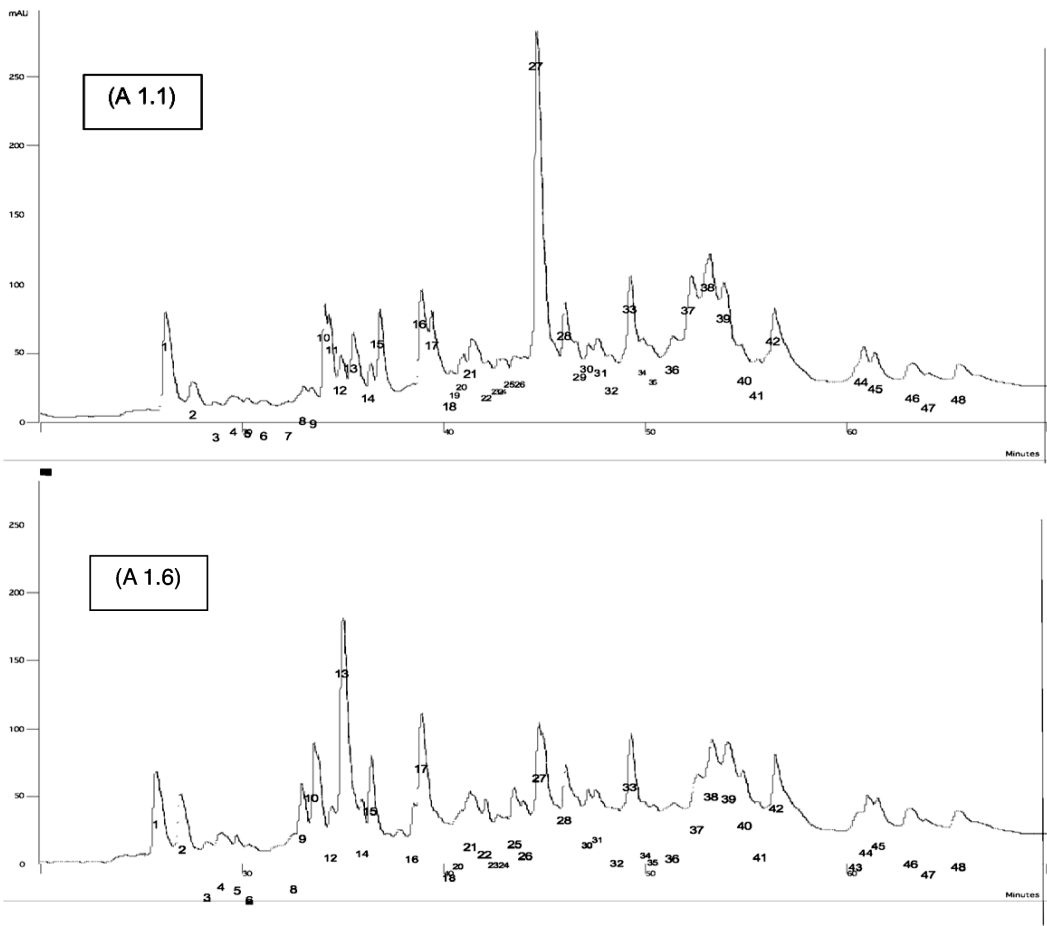


Fig. 7 - RP-HPLC profiles of pH 4.6-soluble fractions of miniature Toma Piemontese cheese made by using the strains *Lactococcus lactis* subsp. *lactis* A 1.1 and A 1.6 after 60 days of ripening.



a washed-curd cheese similar to Saint-Paulin. This study found that Glu, Leu and His were the major FAAs present in cheeses produced with or without the addition of lactobacilli. Cheeses made with the adjunct had higher concentrations of FAA than those made without the adjunct.

MICHAELIDOU *et al.* (2003) studied the effect of a commercial adjunct culture on proteolysis in Feta cheese. The results of their research showed that when strains of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* were added to the normal starter used for cheese production, the level of individual FAAs increased and Leu, Phe and Lys were present in the highest concentrations. HAYALOGU *et al.* (2004) studied the effect of defined strains of *Lactococcus* on proteolysis in Turkish white-brined cheese and found that Leu, Glu, Phe and Lys were the major FAAs pro-

duced by the different strains. POVEDA *et al.* (2004) studied the free amino acid content in Manchego cheese manufactured with different starter cultures and reported that during cheese ripening Glu, Leu, Phe and Lys were present in the highest concentrations. The levels of these FAAs were higher in cheese made with a defined strain-starter or with a defined strain-starter with an adjunct culture than in cheese made using the commercial starter composed of two strains of *Lactococcus lactis*.

## CONCLUSION

The use of different strains of indigenous bacteria significantly influenced proteolysis directly and indirectly. The results of the urea-PAGE of the pH 4.6-insoluble fractions of the cheeses were similar at 30 days of ripening but

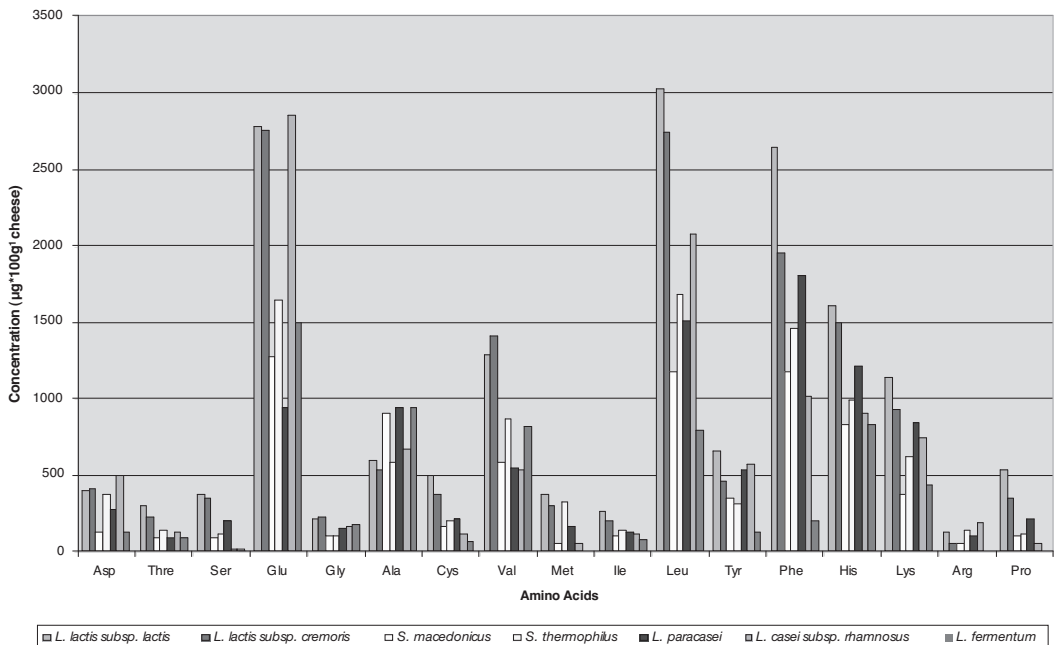


Fig. 8 - Mean concentrations of individual free amino acids after 60 days of ripening in miniature Toma Piemontese cheeses made using different groups of indigenous bacteria as starter.

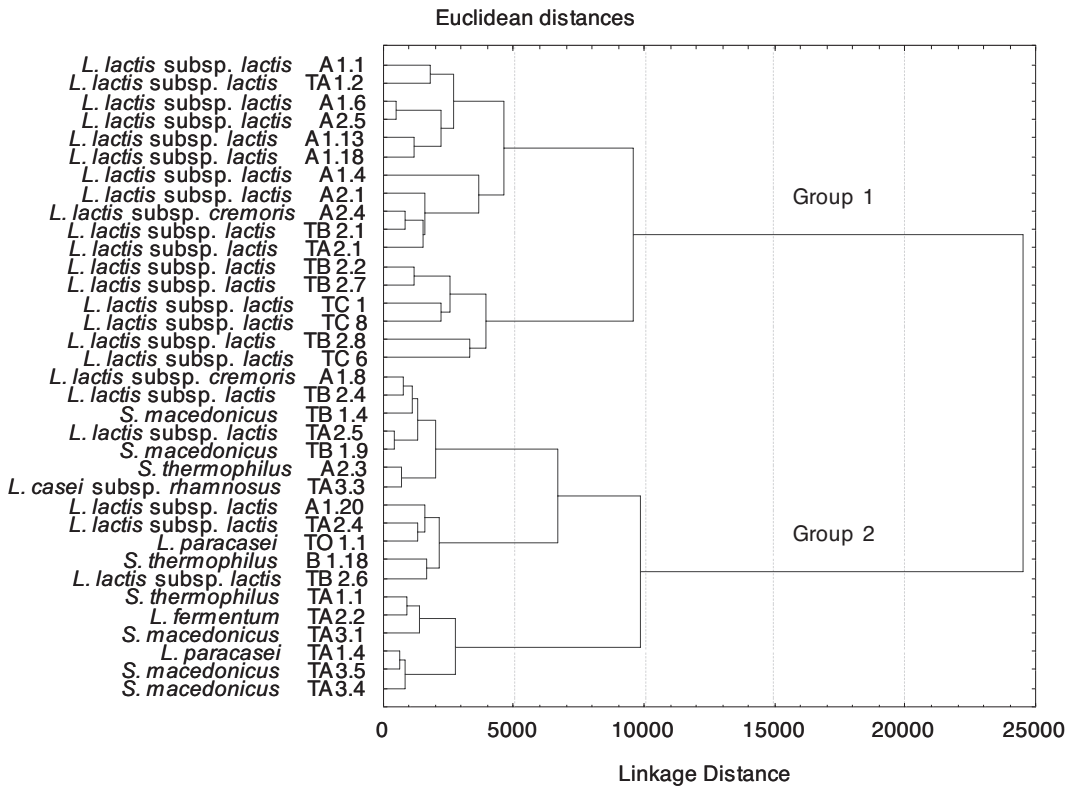


Fig. 9 - Dendrogram from cluster analysis of data obtained from the free amino acid analysis of the cheeses made by using different single strains of lactic acid bacteria after 60 days of ripening.

were very different after 60 days of ripening. Peptide profiles of the pH 4.6-soluble fractions indicated that each strain of indigenous bacteria contributed in a different way to the formation of varying levels of peptides. The different species produced different amounts of the free amino acids. The results of the CA of data obtained after 60 days of ripening, according to the urea-PAGE and the RP-HPLC, showed that the strains were in two groups made up of the same strains except for the strain *Streptococcus macedonicus* TB 1.4.

The data obtained from this study combined with those from other studies could be used to develop new defined-strain starter cultures that could be used in the production of Toma Piemontese cheese.

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