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# CHARACTERIZATION OF AUTOCHTHONOUS LACTIC ACID BACTERIA FROM AN ARTISANAL ITALIAN CHEESE

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## ABSTRACT

We studied the natural lactic bacteria population of an artisanal Italian cheese, Toma piemontese POD, from Piedmont (Northwest Italy), in order to select new strains to be used as starters in large-scale production. Isolates collected from curd and ripened artisanal cheeses were identified by the combined use of PCR 16S-23S rDNA spacer analysis, species-specific probes and 16S rDNA sequencing. Lactococci constitued 67% of the coccal isolates. Enterococci were also isolated together with strains of *Streptococcus macedonicus* and *S. thermophilus*. Lactobacilli were only detected in three samples of curds. Acidification and proteolytic activity and aroma production were also determined for each isolate. On the basis of the results a few isolates were selected and used as starters in cheesemaking trials with both raw and pasteurised milk. The produced cheeses were sensory evaluated and two of them showed typical Toma piemontese taste and flavour. The results suggest the possibility to use these new starters in both dairy industry and artisanal cheesemaking to improve product quality.

Key words: milk products / autochthonous cheese / cheese Toma piemontese / microbiology / lactic acid bacteria / strains / starters / Italy

# KARAKTERIZACIJA MLEČNOKISLINSKIH BAKTERIJ V AVTOHTONEM KMEČKEM ITALIJANSKEM SIRU

## IZVLEČEK

Preučevali smo naravne populacije mlečnokislinskih bakterij v avtohtonem kmečkem italijanskem siru “Toma piemontese POD” iz Piemonta v severozahodni Italiji, da bi pridobili nove seve, ki bi jih lahko uporabljali v industrijski proizvodnji. Izolate, ki smo jih osamili iz sirnine in zrelega avtohtonega sira smo identificirali s pomočjo kombinirane uporabe različnih metod in sicer analizo vmesne regije 16S-23S rDNA z verižno reakcijo s polimerazo, uporabo vrstno specifičnih začetnikov in sekvenciranjem 16S rDNA. Lactococci so predstavljali 67 % vseh izoliranih kokov. Enterokoke smo izolirali skupaj s sevi vrst *Streptococcus macedonicus* in *S. thermophilus*. Laktobacile smo odkrili samo v treh vzorcih sirnine. Za vsak izolat smo ugotavljali tudi proteolitično aktivnost, acidifikacijsko sposobnost in proizvodnjo arome. Na osnovi rezultatov smo izbrali nekatere isolate in jih kot starterske culture uporabili v poskusih izdelave sira in sicer iz surovega in pasteriziranega mleka. Sire smo senzorično ocenili in dva med njimi sta imela za “Toma piemontese» značilen okus in vonj. Naši rezultati nakazujejo možnost izboljšanja kakovosti proizvodov z uporabo teh novih starterskih kultur v sirarski industriji in v kmečki avtohtoni proizvodnji sirov.

Ključne besede: mlečni izdelki / avtohtoni sir / sir Toma piemontese / mikrobiologija / mlečnokislinske bakterije / sevi / starterske kulture / Italija

## INTRODUCTION

In recent years several studies have been carried out to isolate and identify autochthonous lactic bacteria from both raw milk and artisanal cheeses produced with no addition of any starter cultures (Cogan *et al*., 1997; Coppola *et al*., 2001). Increasing information on the natural microbial population present in dairy products can help to prevent the loss of microbial biodiversity in typical foods and consequently the loss of a wide range of cheeses produced by different methods whose typical features depend on local and regional traditions and on the indigenous microbial population present in raw milk and selected by the cheesemaking environment.

Due to cheesemaker's increasing demand for new strains to improve cheese quality, we isolated and identified strains from Toma piemontese POD (Protected Origin Denomination) an artisanal cheese produced in Piedmont (Northwest Italy) to be selected and used as starters in both large-scale and artisanal cheesemaking.

Toma is a semi-cooked cheese which is produced in Piedmont from raw milk warmed to 37–40 °C. Rennet is added at a concentration of 0.15–0.20 mL $L^{-1}$ and the clotting time is established visually by the cheesemaker. The curd is cut into 5–10 mm particles and collected with muslin, pressed and drained for 24 h. Cheese is ripened at 6–10 °C and 85% relative humidity for 30–40 days. The production and the ripening process depend entirely on the natural microbial population present in the milk.

The first objective of this work was to study the natural bacterial population present in the production of Toma piemontese cheese, while the second objective was to select new starters to be used in both artisanal and larger-scale cheesemaking.

## MATERIALS AND METHODS

### Sampling and isolation of bacteria

We collected samples from 7 dairy farms in different regions of Toma piemontese POD production area; 5 curd samples on the day of the production during the summer alpine pasture and 6 cheese samples at 30–40 days of ripening were analysed.

M17 agar medium was used to isolate enterococci, after incubation at 37 °C for 24–48 h and mesophilic and thermophilic cocci, after incubation at 30 °C and 37 °C for 48 h. We used MRS agar pH 5.8 to obtain lattobacilli, after incubation at 30 °C and 37 °C for 48 h.

Randomly selected colonies were purified and then submitted to microscopic examination, Gram staining. The catalase test, production of gas from D-glucose, growth at 6.5% NaCl, at 10 °C and 45 °C were evaluated as well.

### DNA extraction

Genomic DNA for PCR reactions were extracted from 100 μl of an overnight culture diluted with 300 μl of TE 1X buffer (10 mM Tris-HCl, 1 mM $Na_2EDTA$, pH 8.0) as described by Mora *et al.* (2000).

### Identification of isolates

The isolates were identified by the combined use of PCR 16S-23S rDNA spacer analysis (RSA), species-specific PCR and 16S rDNA sequencing according to the methods suggested by the following authors: Jensen *et al.* (1993) for RSA analysis; Ke *et al.* (1999) for species-specific PCR on enterococci, Cheng *et al.* (1997) on *Enterococcus faecium*, Dutka-Malen *et al.* (1995) on

*E. faecalis*, Lick *et al.* (1996) on *Streptococcus thermophilus*, Corroler *et al.* (1999) on *Lactococcus lactis*, Zlotkin *et al.* (1998) on *L. garvieae*, Ward and Timmins (1999) on *Lactobacillus casei*, *L. paracasei* and *L. rhamnosus*, Berthier and Ehrlich (1998) on *L. curvatus* and *L. sakei*.

A 500 bp portion of the 16S rRNA gene was sequenced for some isolates. Amplification was performed according to the protocol used by Lane (1991). PCR products were purified and sequenced using the dideoxy chain-termination principle (Sanger *et al*., 1977). Taxonomic identification was performed using the Ribosomal Database Project (RPD-II) (Maidak *et al*., 2001).

### Aroma analysis

Aroma analysis was performed as follows: 3 g of inoculated milk for each isolate, 10 mL of internal standard (1-heptanol: 10 μg $mL^{-1}$) and 28% (w/w) of NaCl were mixed and stirred at 42 °C for 40 min.

Extraction was carried out at 42 °C for 20 min in head space by SPME by DVB/Carboxen/PDMS fiber (2 cm) and desorbtion at 270 °C for 4 min in splitless.

The analysis was carried out by DB-WAX capillary column according to the following operation conditions: 35 °C for 5 min; 2 °C/min to 183 °C; 5 °C/min to 210 °C; 3 min at 210 °C. Mass spectra was recorded in TIC mode, ionisation voltage of 70 eV and 33–300 amu mass range.

### Acidification activity

In order to evaluate acidification activity of the isolates, we inoculated milk at 2% for each strain and pH measures were taken for 24 h, at incubation temperature of 37 °C.

### Protease activity

Protease activity of the isolates was observed for each strain inoculated in milk at 2% and incubated at 37 °C for 24 h, by colorimetric determination with the Hull method.

### Cheesemaking trials

A few isolates were selected and used as starter in cheesmaking trials.

Eight cheesemakings were carried out on pasteurized cow milk by using 7 different starter mixtures of lactic bacteria isolates and one commercial starter.

Two cheesemakings were carried out on raw cow milk by using starter n.7 and without the addition of any starter culture.

Sensory evaluation was carried out on Toma cheese obtained at 60 days ripening.

## RESULTS

### Isolates

Altogether 116 coccal isolates were collected, 53 from curd samples and 63 from cheese samples (Table 1). The viable counts on M17 plates varied from $10^5$ to $10^6$ cfu $g^{-1}$ for curd samples; higher levels were reached in cheese samples, $10^8$ cfu $g^{-1}$. Lactobacilli were only detected in three samples and their incidence was very low, 1 to 7 cfu $g^{-1}$.

Table 1. Identification of cocci from Toma samples
Preglednica 1. Identifikacija kokov v siru Toma

| Identification | Number of strains |
|---|---|
| *L. lactis* subsp. *lactis* | 24 |
| *L. lactis* subsp. *cremoris* | 10 |
| *L. garvieae* | 44 |
| *S. suis* | 6 |
| *S. agalactiae* | 2 |
| *S. dysgalactiae* | 2 |
| *S. macedonicus* | 6 |
| *S. thermophilus* | 3 |
| *S. uberis* | 1 |
| *E. faecium* | 9 |
| *E. durans* | 4 |
| *E. faecalis* | 2 |
| *Enterococcus* spp. | 4 |

**Identification of isolates**

On the basis of PCR amplification of the 16S-23S rRNA spacer region (RSA) it was possible to cluster the coccal isolates in 5 groups (Fig. 1).

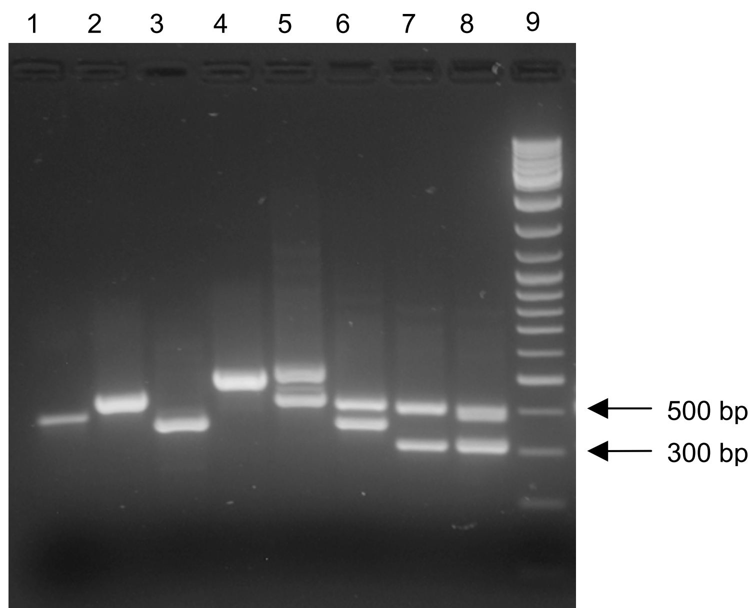


Figure 1. RSA profiles of representative coccal strains of each cluster obtained. Lanes 1–4: cluster I, II, III, IV; lanes 5–8: cluster V, subgroups a, b, c, d; lane 9: DNA Ladder Mix.
Slika 1. Profili regije med rRNA geni pri izbranih sevih, ki predstavljajo posamezne skupine kokov. Steze 1–4: skupine I, II, III in IV; steze 5–8: podskupine a, b, c in d; steza 9: DNA standard.

The isolates grouped in cluster I showed typical spacer region referable to *Lactococcus* lactis, the cocci belonging to cluster II showed a typical spacer region referable to *L. garvieae*, the ones of group III to *S. thermophilus*, the ones of group V to genera *Enterococcus*. In cluster IV six isolates were grouped showing a band at 500 bp.

16S rRNA sequence analysis was necessary to identify isolates included in cluster IV, for which no indication was obtainable by RSA analysis. The analysis of the 16S rRNA sequence together with species-specific PCR was also necessary to identify some dubious isolates belonging to the other groups.

Specific PCR and RSA analysis were also used to identify lactobacilli: three strains belonged to the species *Lactobacillus fermentum*, one to *L. coryniformis*, two to *L. paracasei*, two to *L. curvatus* and two to *L. rhamnosus*.

**Aroma analysis**

Genetically identified isolates were submitted to aroma analysis; they were chosen among the strains obtained from curd and belonging to the species *L. lactis* subsp. *lactis*, *L. lactis* subsp. *cremoris*, *S. thermophilus*, *L. paracasei*,

GC-MS analysis highlighted the presence of about 100 organic compounds mainly belonging to aldehydes, alcohols, acids, of which over 40 are not present in the milk.

The processing of these data by Principal Component Analysis allowed us to group these isolates in 4 sets by using the first three components (Fig. 2).

A group is formed by B18 strains belonging to the species *S. thermophilus*. *L. lactis* supsp. *lactis* strains A4, A1, A18 and A13, A20, A6 form respectively the second and third group. The fourth group is constituted by the only *L. lactis* supsp. *cremoris* A8 strains.

Milk aroma is quite different therefore it is located in a different space (M) of the score plot.

Figure 2. Score plots of the first three PCA (Principal Component Analysis) factors performed on the basis of the concentration of aromatic compounds produced by lactic acid bacteria strains.

Slika 2. Predstavitev vrednosti prvih treh dejavnikov (analiza glavnih komponent) na osnovi koncentracij aromatskih sestavin, ki jih proizvajajo sevi mlečnokislinskih bakterij.

## Starters

The selection of strains to be used as starters in cheesemaking trials was carried out by the choice of, at least, one isolate for each PCA group. For each group was considered the strain showing quicker acidification activity in the 24 h and higher protease activity. One *L. paracasei* strain has been added to starters n.2, n.4, n.6 for the high acidification and proteolytic activities showed. The bacterial composition of the seven starter mixes, chosen according to the above criteria and to the results obtained, is shown in Table 2.

Generally each mix contained only two strains with the exception of starter n. 7 composed of five different strains. The mix combination of two strains of *L. lactis* supsp. *lactis* has not been considered while a commercial starter, generally used for Toma piemontese POD cheesemaking, was used.

## Cheesemaking trials

We obtained the best sensory results from n.7 in trials with both pasteurized and raw cow milk. In both cases cheese produced from starter n.7 was better in structure, taste and aroma than cheese without the addition of any starter.

Table 2. Starter compositions used in cheesemaking trials
Preglednica 2. Sestava starterskih kultur, ki so bile uporabljene v sirarskem poskusu

| Starter | Isolates |
|---|---|
| n.1 | B18 (*S. thermophilus*) – A18 (*L. lactis* subsp. *cremoris*) |
| n.2 | G1 (*Lb. paracasei*) – A18 (*L. lactis* subsp. *cremoris*) |
| n.3 | A6 (*L. lactis* subsp. *lactis*) – B18 (*S. thermophilus)* |
| n.4 | A6 (*L. lactis* subsp. *lactis*) – G1 *(Lb. paracasei)* |
| n.5 | A8 *(L. lactis* subsp. *lactis)* – B18 *(S. thermophilus)* |
| n.6 | A8 *(L. lactis* subsp. *lactis)* –G1 *(Lb. paracasei*) |
| n.7 | A6 *(L. lactis* subsp. *lactis)* – A8 *(L. lactis* subsp. *lactis)* – A18 (*L. lactis* subsp. *cremoris*) – B18 *(S. thermophilus)* – G1 *(Lb. paracasei*) |
| n.8 | Commercial starter |

Toma cheese obtained by using starter n.7 in raw milk compared to the one without starter is shown in Fig. 3. The relative sensory evaluations showed that the first one is a good product characterized by typical aroma and taste, proper eyes and structure while the other one showed defective structure and strong and bitter aroma due to lipolysis and proteolysis.

## CONCLUSIONS

The results of the present work represent the first approach to understanding the bacterial population involved in traditional Toma piemontese POD cheese.

The technological performance of these strains suggest the possibility of their use in the production of Toma piemontese POD cheese in order to improve and standardize product quality.

Further investigations to prove the technological characteristics of these strains and their stability will be needed.

New cheesemakings are still in progress to test new starter combinations.

Figure 3. Toma cheese produced from raw cow milk using starter n.7 (a) and with no addition of any starter (b).

Slika 3. Sir Toma, izdelan iz surovega mleka ob uporabi starterske kulture št. 7 (a) in brez starterske culture (b).

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