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Pecorino Crotonese cheese: Study of bacterial population and flavour compounds

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ABSTRACT

The diversity and dynamics of the dominant bacterial population during the manufacture and the ripening of two artisanal Pecorino Crotonese cheeses, provided by different farms, were investigated by the combination of culture-dependent and -independent approaches. Three hundred and thirty-three strains were isolated from selective culture media, clustered using Restriction Fragment Length Polymorphism and were identified by 16S rRNA gene sequencing. The results indicate a decrease in biodiversity during ripening, revealing the presence of *Lactococcus lactis* and *Streptococcus thermophilus* species in the curd and in aged cheese samples and the occurrence of several lactobacilli throughout cheese ripening, with the dominance of *Lactobacillus rhamnosus* species. Bacterial dynamics determined by Denaturant Gradient Gel Electrophoresis provided a more precise description of the distribution of bacteria, highlighting differences in the bacterial community among cheese samples, and allowed to detect *Lactobacillus plantarum*, *Lactobacillus buchneri* and *Leuconostoc mesenteroides* species, which were not isolated. Moreover, the concentration of flavour compounds produced throughout cheese ripening was investigated and related to lactic acid bacteria presence. Fifty-seven compounds were identified in the volatile fraction of Pecorino Crotonese cheeses by Gas Chromatography–Mass Spectrometry. Esters, alcohols and free fatty acids were the most abundant compounds, while aldehydes and hydrocarbons were present at low levels.

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1. Introduction

The occurrence of bacterial population, especially of lactic acid bacteria (LAB), during manufacture and ripening of most cheese varieties is already well documented (Beresford et al., 2001; Wouters et al., 2002). The origin of microorganisms may vary, entering from milk and/or with other ingredients used in cheese-making, or adventitiously from the environment, and LAB are considered the microrganisms mainly involved in flavour formation of cheese variety (Fox et al., 1996). It is noteworthy that the development of unique flavours in cheese is the result of complex reactions, e.g. glycolysis, lypolysis and proteolysis, mainly due to enzymes from milk, rennet and microorganisms (Fox, 1989). The proteolysis is undoubtedly the most important biochemical process for flavour and texture properties of semi-hard and hard cheese types. Proteolytic enzymes from LAB play an important role in the degradation of casein and peptides leading to the production of free amino acids, which are rapidly converted into specific volatile compounds by nonstarter lactic acid bacteria (NSLAB) as well as by lactococci (Ayad et al., 2000; Amarita et al., 2001; Kieronczyk et al., 2003). Several studies have demonstrated the occurrence of LAB species in several Italian cheeses like Canestrato Pugliese (Aquilanti et al., 2006), Parmigiano Reggiano (Gala et al., 2008), Pecorino (De Angelis et al., 2001; Randazzo et al., 2006, 2008), Ragusano (Randazzo et al., 2002), Raschera and Castelmagno (Dolci et al., 2008a,b), Provola dei Nebrodi (Cronin et al., 2007), Fontina (Giannino et al., 2009); in several Spanish artisanal starter-free cheese types (Oneca et al., 2003; Sánchez et al., 2006; Abriouel et al., 2008; Martín-Platero et al., 2008), and in French cheeses (Duthoit et al., 2003; Callon et al., 2004). Up to now no information is available on the composition of the bacterial population and on flavour formation throughout cheese manufacture and ripening of Pecorino Crotonese (PC) cheese.

Pecorino Crotonese is an artisanal cheese manufactured on a small scale by farmers, following traditional practices, in a well-defined area of Southern Calabria (Italy). It is produced from raw ewes' milk with the addition of kid rennet paste. According to a traditional protocol, the use of starter culture is not allowed and the acidification is due to the autochthonous lactic acid bacteria (LAB). Hence, the quality of raw milk, the environmental conditions and the traditional manufacture play a major role in determining the characteristics of this artisanal PC cheese and have a clear effect on the microbial population. Characterizing cheese microbial population may contribute to understand the ecological processes that drive microbial interaction in cheese and their technological relevance.

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At present, a wide range of molecular approaches is available to study bacterial community in cheese, including culture-dependent and -independent techniques.

Culture-dependent techniques, based on cultivation followed by phenotypic and molecular identification, are known to be laborious and time-consuming to monitor population dynamics and may over- or underestimate the microbial diversity (Randazzo et al., 2002; Ercolini et al., 2004). In the last decade, the profiling of bacterial populations became more precise with the application of molecular techniques based on the direct detection of DNA and RNA in microbial ecosystems. The application of the Denaturing Gradient Gel Electrophoresis (DGGE) of the 16S rRNA gene to study microbial communities and to monitor their dynamics during manufacture and ripening of artisanal cheeses has recently been reviewed (Randazzo et al., 2009).

The aim of the present study was to evaluate bacterial population of PC cheese through a combination of culture-dependent and -independent approaches, in order to obtain a complete description of the dominant species involved during manufacture and ripening and to assess their contribution to flavour formation by Solid Phase Micro Extraction (SPME) and Gas Chromatography–Mass Spectrometry (GC–MS) analyses.

2. Materials and methods

2.1. Cheese-making procedure and sampling

The Pecorino Crotonese cheese-making procedure is already documented (Gardini et al., 2006). Two kinds of PC cheese are currently produced: semi-ripened (60-days-old) and ripened (up to 2 years-old) one. The cheese samples used in this study were collected from two farmers (A and B) from two different areas of Crotone (Calabria, Italy), and the cheeses were chosen based on their high quality properties and collected in two-consecutive weeks. Curd, semi-ripened (60 days) and ripened cheese (120 days) samples were aseptically taken, in duplicate, during cheese manufacture and ripening, and they were subjected to bacteriological analysis within 6 h or stored at −80 °C.

2.2. LAB reference strains and culture conditions

The LAB reference strains *Enterococcus faecalis* DSM #20468^T, *Enterococcus faecium* DSM #20478^T, *Enterococcus hirae* DSM #20160^T, *Lactobacillus brevis* DSM #20054^T, *Lactobacillus buchneri* DSM #20057^T, *Lactobacillus delbrueckii* subsp. *lactis* DSM #20072^T, *Lactobacillus fermentum* DSM #20052^T, *Lactobacillus helveticus* DSM #20075^T, *Lactobacillus paracasei* subsp. *paracasei* DSM #5622^T, *Lactobacillus pentosus* DSM #20314^T, *Lactobacillus plantarum* DSM #20246^T, *L. plantarum* subsp. *plantarum* DSM #20174^T, *Lactobacillus rhamnosus* DSM #20021^T, *L. rhamnosus* GG, *Lactococcus lactis* subsp. *lactis* DSM #20481^T, *Streptococcus thermophilus* DSM #20617^T used in this study came from the Deutsche Sammlung von Mikroorganismen und Zelkulturen (Braunschweig, Germany). Dairy wild strains *Lactobacillus curvatus* RC23, *Lactobacillus paraplantarum* F3, *L. lactis* subsp. *cremoris* LC1, and *Leuconostoc mesenteroides* CR310 were taken from DOFATA microbial collection. Lactococci, enterococci and streptococci were cultivated on LM17 medium, M17 medium (Oxoid, Basingtoke, United Kingdom) supplemented with 5 g l^{-1} of lactose, and *Leuconostocs* on MRS agar (Oxoid). Incubation was performed at 32 °C and 42 °C for 24–48 h for mesophilic and thermophilic bacteria, respectively, under anaerobic conditions using an Anaerogen kit (Oxoid, Milano, Italy).

2.3. Enumeration and isolation of LAB

Samples (10 g) of curd were taken directly during cheese-making and two diametrically opposed samples (10 g) of semi-ripened and ripened cheeses (60 and 120 days, respectively) including either the cheese core or surface were cut up, ground in a sterile food mill, pooled, serially diluted in 90 ml sterile physiological solution (0.9% NaCl), and homogenized with a Stomacher Lab-Blender 400 (Seward Medical, London, United Kingdom) for approximately 5 min. The samples were analyzed by plating appropriate ten-fold dilutions onto the following media: PCA (Oxoid,) for mesophilic aerobic bacteria; MRS (Oxoid), acidified to pH 5.4 with hydrochloric acid (HCl, 1 M); Rogosa agar (Oxoid) for mesophilic and thermophilic lactobacilli; LM17 medium containing cycloheximide (Fluka Chimica, Milan, Italy) (100 μg l^{-1} added after sterilization) for *Lactococcus* and *Streptococcus*; MSE agar (Biolife, Milan, Italy) for *Leuconostoc*; and KAA agar base (Kanamycin Aesculin Azide, Oxoid), containing Kanamycin Selective Supplement (Oxoid), for enterococci. Plates containing MRS and KAA agar media were incubated under anaerobic conditions using an Aerogen kit at 37 °C for 48–72 h LM17 plates were incubated at 32 °C and 42 °C for 24–48 h for mesophilic and thermophilic cocci, respectively and plates containing MSE medium were incubated at 30 °C for 48–72 h.

2.4. Phenotypic identification of LAB isolates

To characterize the bacterial population, a representative number of colonies was randomly picked from various agar plates and each colony was purified by streaking three times. All isolates were subsequently cultured on LM17 and MRS agar and finally stored at −20 °C in the same media, containing 20% glycerol, before being subjected to physiological, technological and genotypic identification.

All isolates were characterized by determining their Gram reaction, their catalase activity, spore formation, and ability to grow in MRS broth at 10 °C and 45 °C in stationary tubes. Cell morphology was observed with a phase contrast microscope.

2.5. Technological characterization of LAB strains

The technological properties of LAB strains studied in the present work were: the ability to acidify and to coagulate both ewes' and skim milk powder (Oxoid). The ability of strains to acidify ewes' milk and sterile skim milk powder (reconstituted at 100 g l^{-1}) containing 0.1% of Yeast Extract (Oxoid), was determined using a pH-meter (Eutech Instruments, XSPH 510, Nijkerk, The Netherlands), after 8 h of incubation at 30 °C. The coagulating activity was evaluated by the appearance of visual coagulum on the inner site of glass tube containing both ewes' and powder milk.

2.6. DNA extraction from bacterial strains and from dairy samples

Genomic DNA from bacterial isolates and reference strains was extracted from 6 ml of overnight grown cultures as described by Gala et al. (2008). Total DNA extraction from dairy samples was performed according to the protocol previously described by Randazzo et al. (2002).

2.7. PCR amplification

PCR amplification was performed in a 50 μl volume using a GenAmp PCR System 9700 (Perkin–Elmer, Foster City, CA, USA). The reaction mixtures consisted of 1.25 U of *Taq* DNA polymerase (Invitrogen, Carlsbad, CA, USA), 20 mM Tris HCl (pH 8.4), 50 mM KCl, 3 mM $MgCl_2$, 50 μM dNTPs, 5 pmol primers each and 1 μl of properly diluted template DNA. The reaction mixture with no template DNA was used as a negative control. The universal primers 7-f and 1510-r (Lane, 1991) were used to amplify the 16S ribosomal RNA gene of the isolates and the reference strains. The cycling program was the following: initial denaturation of DNA for 5 min at 94 °C; 35 cycles

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