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Microbial diversity and succession during the manufacture and ripening of traditional, Spanish, blue-veined Cabrales cheese, as determined by PCR-DGGE

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Abstract

The diversity and dynamics of the dominant microbial communities arising during the manufacture and ripening of four batches of naturally fermented Cabrales cheese were investigated by the PCR-DGGE culture-independent technique. Total microbial DNA was extracted from cheese milk, curd and cheese samples and used as template material in PCR experiments to amplify the V3 region of the bacterial 16S rRNA gene, plus the D1 region of the eukaryotic 26S rRNA gene. These regions were then analysed using DGGE. Eukaryotic and bacterial bands were identified by isolation, reamplification and sequencing. The results were compared to those obtained in a previous microbial characterization of the same four batches using classical culturing methods. Great variability was recorded between batches by the PCR-DGGE technique. This was also shown by culturing, and underlines the uniqueness of artisanal products. *Lactococcus lactis* subsp. *lactis* was dominant from the cheese milk stage until the end of ripening, whereas populations of certain *Lactobacillus* species appeared during ripening. Populations of species never isolated by culturing were found to be numerous by the PCR-DGGE method, in particular *Lactococcus garvieae* and *Lactococcus raffinolactis*. Other, completely unknown lactococci were also detected. The dominant eukaryotic populations from day 15 onwards were those of *Penicillium roqueforti* and *Geotrichum candidum*.

Keywords: Traditional cheeses; Blue-veined cheese; Cabrales cheese; *Penicillium roqueforti*; DGGE; Culture-independent microbiology

1. Introduction

Cabrales is the most famous of the traditional, Spanish, blue-veined cheeses, and has enjoyed Protected Designation of Origin (PDO) status since 1981. It is made in the mountainous area of the *Picos de Europa* (Principality of Asturias, Northern Spain) from cow's milk, with seasonal additions of goat's and sheep's milk. Its traditional manufacture involves curdling mixtures of evening and morning milk at 28–30 °C with farm-made goat rennet, but does not involve the addition of starter cultures or mould spores (Núñez, 1978). The curd is then cut into pieces the size of hazelnuts and placed in cylindrical moulds at room temperature. The cheeses are turned upside down several times for whey drainage without pressing, after which they are covered in coarse salt and kept at room temperature for around 10–15 days. Finally, the dry cheeses are placed in natural caves in the production area, where ripening takes place at a nearly constant relative humidity (90–95%) and temperature (9–12 °C). Under these conditions, *Penicillium roqueforti* enters the cheese and develops in the matrix, providing the final product its characteristic appearance. Microbiologically, this cheese offers a complex habitat in which prokaryotic and eukaryotic populations interact and develop throughout manufacturing and ripening. As a result, it acquires the unique organoleptic qualities afforded by the local environment (which includes the presence of the adventitious microbiota that grows during these processes). Different characteristics of its cultured microbial populations have been recorded (Núñez, 1978; Núñez and Medina, 1980; Núñez et al., 1981; Flórez et al., 2006a).

Denaturing gradient gel electrophoresis (DGGE) and its relative, temperature gradient gel electrophoresis (TGGE), are commonly used to study dairy products and dairy environments (Ercolini et al., 2001; Cocolin et al., 2004; Lafarge et al., 2004),

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and have provided static descriptions of cheese-associated bacteria (Coppola et al., 2001; Ogier et al., 2002; Ercolini et al., 2003; Ogier et al., 2004). The DGGE technique has even been used to characterize the yeast population profiles of raw milk (Cocolin et al., 2002). Despite promising results, the use of these techniques has only been applied to study the diversity and dynamics of the microbial populations in traditional cheeses of the stretched curd group (Mozzarella-type) (Coppola et al., 2001; Randazzo et al., 2002; Ercolini et al., 2004). Using the DGGE method, Randazzo et al. (2002) found that the *Lactobacillus delbrueckii* population was among the most dominant and metabolically active during the ripening of Ragusano cheese, yet this species has never been isolated on selective media.

This paper reports the microbial characterization of Cabrales cheese by the culture-independent PCR-DGGE technique. This study was undertaken to improve our understanding of the composition and dynamics of the dominant microbial populations arising during manufacture and ripening. The results were compared to those previously obtained by a classical culture-based methodology (Flórez et al., 2006a). The information gathered may be useful in the selection of commercial starters or in the design of specific cultures for this cheese.

2. Material and methods

2.1. Cheese manufacture and sampling

Four batches of *Cabrales* cheese were made by two manufacturers at different times of the year. Producer I followed the traditional manufacturing method, including the use of artisanal kid rennet extract for curdling the milk, and avoiding the use of starter or mould spores (batches A and B). In contrast, Producer II used commercial calf rennet and inoculated the milk with commercial *P. roqueforti* spores (Strain PB6; Christian-Hansen, Hølstrom, Denmark) (approximately 1×10^6 cfu of moulds ml^{-1}; batches C and D). Samples were taken from the cheese milk, curd and cheeses at 3, 7, 15, 30, 60 and 90 days of ripening, following the standard FIL-IDF procedure (Anonymous, 1985). To sample the curd and cheese, cubes of 10 g were procured from the inside of the products and homogenized with 45 ml of 2% (w/v) sterilized sodium citrate solution at 45 °C for 1 min in a Colworth Stomacher 400 (Seward Ltd., London, UK).

2.2. DNA extraction from cultured bacteria

Total DNA from representative lactic acid bacteria was isolated as described by Leenhouts et al. (1988). Briefly, cells grown in 10 ml of either M17 or MRS (both from VWR International, Darmstadt, Germany) supplemented with 40 mmol l^{-1} DL-threonine (Sigma, Sigma-Aldrich Co., St. Louis, Miss., USA) were suspended in 0.5 ml of a lysis solution (25 mmol l^{-1} Tris hydrochloride, pH 8.0, 50 mmol l^{-1} EDTA, 50 mmol l^{-1} glucose) (all reagents from VWR International) containing 30 μg ml^{-1} of lysozyme (USB; Amersham Biosciences Europe GmbH, Cerdanyola, Spain) and 150 U of mutanolysin (USB), and incubated for 1 h at 37 °C. Two hundred microlitres of 10% (v/v) sodium dodecyl sulphate (Sigma) and 15 μl of proteinase K (Roche Applied Science, Penzberg, Germany) (20 mg ml^{-1}) were then added, and incubation allowed to proceed at 60 °C until the suspension was clear. The lysate was finally extracted with phenol–chloroform–isoamyl alcohol (24:24:1) (USB). A quantity of sodium acetate (Sigma) (3 M) equal to 1/10 of the volume of the water phase was then added, and the DNA precipitated with two volumes of 96% ethanol (VWR International). After centrifugation (15,000 ×*g* for 10 min), the pellet was washed with 70% ethanol and, after drying, the DNA was dissolved in 50 μl of TE (10 mmol l^{-1} Tris hydrochloride, 1 mmol l^{-1} EDTA, pH 8.0) supplemented with 5 μg ml^{-1} of RNase (Roche).

2.3. Isolation of microbial DNA from milk and cheese samples

Five hundred microlitres of pronase (Sigma) solution (100 mg ml^{-1}) and 100 μl of β-mercaptoethanol (VWR International) were added to the cheese milk samples (40 ml) and cheese homogenates and incubation allowed to proceed at 37 °C for 3 h. The cells were then pelleted from the supernatants by centrifugation and washed twice with a lysis buffer (20% sucrose, 10 mmol l^{-1} Tris–HCl, 510 mmol l^{-1} EDTA, 50 mmol l^{-1} NaCl, pH 8.0). They were then disrupted with glass beads (106 μm diameter) (Sigma) using a Minibead Beater apparatus (Biospec Products Inc., Bartlesville, Ok., USA) at 5000 rpm for 1 min (repeated three times). The DNA was finally purified by phenol–chloroform–isoamyl alcohol extractions and precipitated by ethanol. It was then suspended in sterile water containing 5 μg ml^{-1} of RNase.

2.4. PCR amplification and DGGE conditions

Total DNA from the cheese milk and cheese samples was used as template material to amplify the V3 region of the bacterial 16S rRNA gene by PCR, using the universal primers F357 (5′-TACGGGAGGCAGCAG-3′) and R518 (5′-ATTACCGCGGCTGCTGG-3′) (Muyzer et al., 1993). A GC clamp (5′-CGCCCGCCGCGCGCGGCGGGCGGGGCGGG-GGCACGGGGG-3′) was linked to the first primer to obtain F357-GC. For eukaryotic organisms, the D1 domain of the 26S rRNA gene was amplified under the same conditions with primer NL1 (5′-GCCATATCAATAAGCGGAGGAAAAG-3′), to which the GC clamp was linked to render NL1-GC and LS2 (5′-ATTCCCAAACAACTCGACTC-3′) (Cocolin et al., 2002).

Primers were all supplied by Genosys (Sigma), and PCR was performed in 50 μl volumes containing 10 mmol l^{-1} Tris–HCl, 50 mmol l^{-1} KCl, 1.5 mmol l^{-1} $MgCl_2$, 0.2 mmol l^{-1} of each dNTPs, 0.2 m mol l^{-1} of the primers, 1.5 U of *Taq*-polymerase (Roche Diagnostics, Barcelona, Spain) and 2 μl of extracted DNA. The PCR conditions for amplification of prokaryotic and eukaryotic sequences were essentially those described by Muyzer et al. (1993) and Cocolin et al. (2002), respectively.

DGGE was performed in a DCode apparatus (Bio-Rad, Richmond, Ca., USA) at 60 °C on 8% polyacrylamide gels (Bio-Rad) with formamide-urea (Bio-Rad) denaturing ranges of 40–60% and 30–50% for bacteria and fungi, respectively. The