Assessment of microbial populations dynamics in a blue cheese by culturing and denaturing gradient gel electrophoresis (DGGE)

Ángel Alegría¹, Renata González², Mario Díaz², and Baltasar Mayo^{1*}

¹Departamento de Microbiología y Bioquímica, Instituto de Productos Lácteos de Asturias (CSIC), Carretera de Infiesto, s/n, 33300-Villaviciosa, Spain, and ²Departamento de Ingeniería Química y Tecnología del Medio Ambiente, Facultad de Químicas, Universidad de Oviedo, C/ Julián Clavería, s/n, 33006-Oviedo, Spain

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*Corresponding author:

Baltasar Mayo, ¹Departamento de Microbiología y Bioquímica, Instituto de Productos Lácteos de Asturias (CSIC), Carretera de Infiesto s/n, 33300-Villaviciosa, Spain

Tel.: 34+985 89 21 31

Fax: 34+985 89 22 33

E-mail address: baltasar.mayo@ipla.csic.es

Summary

The composition and development of microbial population during the manufacture and ripening of two batches of a blue-veined cheese was examined by culturing and polymerase chain reaction (PCR) denaturing gradient gel electrophoresis (DGGE) (PCR-DGGE). Nine selective and/or differential media were used to track the cultivable populations of total and indicator microbial groups. For PCR-DGGE, the V3 hyper variable region of the bacterial 16S rRNA gene and the eukaryotic D1 domain of 28S rDNA were amplified with universal primers, specific for prokaryotes and eukaryotes, respectively. Similarities and differences between the results obtained by the culturing and the molecular method were recorded for some populations. Culturing analysis allows minority microbial groups (coliforms, staphylococci) to be monitored, although in this work PCR-DGGE identified a population of *Streptococcus thermophilus* that went undetected by culturing. These results show that the characterization of the microbial populations interacting and evolving during the cheesemaking process is improved by combining culturing and molecular methods.

Introduction

The microbial characterization of dairy products has traditionally been performed using culture dependent techniques employing non-selective, selective and differential media; this allows the composition and development of majority and indicator populations present during manufacturing and ripening to be monitored. However, a vast array of culture-independent molecular methods is now being used for the microbial typing of food and food fermentations [10, 11]. Molecular methods overcome culture-associated drawbacks such as the low specificity of selective media and the inability to enumerate cells in a viable but non-cultivable state. Most molecular methods rely on the amplification of rDNA or rRNA sequences by the polymerase chain reaction (PCR) and the subsequent electrophoretic analysis of the amplicons produced [1]. In addition, they are less laborious, faster and cheaper than culture-based analyses. However, these techniques are not free from bias nor without limitations [20] and are generally thought of as complementary to conventional methods, providing a more precise microbial picture of food ecosystems.

Denaturing gradient gel electrophoresis (DGGE) and its relative, temperature gradient gel electrophoresis (TGGE), were developed to analyse microbial communities based on sequence-specific distinctions of 16S rRNA amplicons produced by PCR [15]. Separation is based on the reduced electrophoretic mobility of partially melted double-stranded DNA molecules in polyacrylamide gels with a linear gradient of denaturing agents (urea and formamide) or temperature. A GC clamp of around 50 bp is attached to the 5' end of one of the primers, preventing the two DNA strands from undergoing complete disassociation. If the total DNA of a microbial community is used in PCR amplification these techniques can provide the profile of the genetic diversity of the dominant populations. If total RNA is used

instead, the profiles reveal the metabolically-active populations [14]. Both PCR-DGGE and PCR-TGGE have recently been used to study different microbial aspects of food-related environments [7], including the diversity and dynamics of microorganisms present during cheese manufacture and ripening [4, 5, 6, 8, 16].

The present paper reports the use of plate count methods and PCR-DGGE analysis for studying microbial development, diversity and evolution during the manufacture and ripening of a blue cheese made from pasteurized milk inoculated with commercial starters (mesophilic lactic acid bacteria as acidifiers and *Penicillium roqueforti* spores as the ripening culture). The aim of this work was to compare the results obtained with the culture-dependent and culture-independent methods, assessing the advantages and drawbacks of each.

Materials and Methods

Cheese manufacture and sampling conditions

Two batches of blue-veined cheese were manufactured from pasteurized milk under standard conditions [9]. Acidifying starters (Flora Danica) and ripening cultures (*Penicillium roqueforti* strains PR3 and PRG3) were purchased from Chr. Hansen (Hørsholm, Denmark). Milk, curd and cheese were sampled according to FIL-IDF standard 50B and transported to the laboratory under refrigerated conditions. Culturing analyses were performed on the day of sampling. For the isolation of DNA, milk, curd and cheese samples were stored at -20°C until required.

Determination of microbial composition by plate counting

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Ten milliliters of milk or 10 g of curd and cheese were homogenized with 90 mL of a 2 % (w/v) sodium citrate solution at 45 °C in a Colworth Stomacher 400 (Seward Ltd., London, UK) (for 3 x 1 min). Ten-fold serial dilutions were made in Maximum Recovery Diluent (Scharlab, Barcelona, Spain) and plated in duplicate onto general and selective media, as follows.

Aerobic mesophilic counts. Aerobic mesophilic bacteria were counted on Plate Count Agar supplemented with 0.1 % skimmed milk (PCA; Merck, Darmstadt, Germany) after 72 h of incubation in aerobiosis at 30 °C.

Lactococci: Lactococci were grown on M17 agar (Scharlab) and enumerated after 48 h of incubation at 30 °C.

Lactobacilli. Lactobacilli were grown on de Man, Rogosa and Sharpe agar (MRS; Merck), adjusted to pH=5.4 and enumerated after 72 h of incubation at 32 °C in a 5 % CO₂ enriched incubator.

Leuconostoc spp. Dextran-producing leuconostocs were grown on Mayeux, Sandine and Elliker agar (MSE; Biokar Diagnostics, Beauvais, France) and enumerated after five days of incubation at 25 °C.

Enterococci. Enterococci were grown on Slanetz and Bartley agar (S-B; Merck) and enumerated after 24 h of incubation at 44 °C.

Enterobacteria and coliforms. Enterobacteria and coliforms were grown on Violet Red Bile Glucose agar (VRBG) and Violet Red Bile Lactose agar (VRBL) (both from Merck) respectively, using the pour-plate and overlay technique. In brief, dilutions were mixed with 15 mL of agar and poured onto Petri dishes. After solidification, a second agar layer of 10 mL was added. Bacteria were enumerated after 48 h of incubation at 30 °C.

Staphylococci. Dilutions were grown on Baird-Parker agar (B-P; Merck) supplemented with egg yolk tellurite solution (Merck). Black colonies with or without egg yolk clearing were recorded after 24 h of incubation at 37 °C.

Yeasts and moulds. Dilutions of milk, curd and cheese samples were plated on Yeast-Extract Glucose Chloramphenicol agar (YGC; Merck). Yeasts and moulds were independently recorded after 3-5 days of incubation at 25 °C.

Except for the YGC plates, 100 µg/mL of cycloheximide (Merck) was added to all enumeration media to inhibit the growth of moulds and yeasts.

PCR-DGGE analysis

Extraction of total microbial DNA.

Milk, cheese and starter samples homogenized in 2 % sodium citrate were used for the isolation of total microbial DNA. DNA extraction was accomplished using a commercial kit (QIAamp DNA Stool Mini Kit; Quiagen, GmbH, Hilden, Germany), following the supplier's recommendations.

PCR amplification.

Total DNA was used as a template in PCR amplification of the V3 region of the bacterial 16S rRNA gene using the universal primers F357 (5'-TACGGGAGGCAGCAG-3'), to which a 39 bp GC sequence was linked to give rise to GC-F357), and R518 (5'-ATTACCGCGGCTGCTGG-3') [14]. The D1 domain of the 28S rRNA fungal gene was amplified using primers GC-NL1 (5'-GCCATATCAATAAGCGGAGGAAAAG-3') and LS2 (5'-ATTCCCAAACAACCTCGACTC-3') [3]. PCR was performed in a 50 µl volume using a Taq-DNA polymerase master mix (75 mM Tris-HCl pH=8.5, 20 mM (NH₄)₂SO₄, 1.5 mM

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110 MgCl₂, 0.1 % Tween 20[®], 0.2 mM of each dNTP, and 1.25 units Taq polymerase (Ampliqon
111 ApS, Skovlunde, Denmark), with 100 ng of extracted DNA and 0.2 mM of each primer. The
112 amplification conditions for prokaryotic and eukaryotic sequences were those described by
113 Muyzer et al. [14] and Cocolin et al. [3], respectively.

114
115 Electrophoresis conditions.

116 DGGE was performed using a DCode apparatus (Bio-Rad, Richmond, CA., USA) at 60 °C,
117 employing 8 % polyacrylamide gels with a denaturing range of 40–60 % for bacteria and 30–
118 50 % for fungi. Electrophoresis was performed at 75 V for 17 h and at 130 V for 4.5 h for
119 bacterial and fungal amplifications respectively. Bands were visualized by staining with 0.5
120 µg/mL ethidium bromide (Sigma-Aldrich, St. Louis, MO, USA).

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122 Identification of PCR-DGGE bands.

123 DNA bands in the polyacrylamide gels were assigned to species by either comparison with a
124 control ladder of known strains [8], or by sequencing and comparison of the sequences after
125 isolation of DNA from the bands and re-amplification with the same primers without the GC-
126 clamps. Sequences with a percentage identity of 97% or greater were considered to belong to
127 the same species (Stackebrandt and Goebel, 1994).

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129 **Results and Discussion**

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131 Microbial counts using conventional plate count techniques

132 Tables 1 and 2 show the enumeration results for total and indicator populations in the
133 two batches over manufacturing and ripening. As expected for a cheese made from

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5 134 pasteurized milk, *Enterobacteriaceae*, coliforms, enterococci and staphylococci counts were
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7 135 always in low numbers, though small variations between batches were recorded. No
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9 136 *Staphylococcus aureus* strains were detected. Lactococci were found in the largest numbers;
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11 137 in M17 the numbers recorded usually matched those of total bacterial aerobic counts in PCA,
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13 138 reaching over 10^9 colony forming units (CFU)/g between day 3 and day 7 (Tables 1 and 2).
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15 139 Lactobacilli grew from low numbers in milk up to 10^8 CFU/g by day 7, although their final
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17 140 numbers were one log unit lower than those of lactococci. Both lactococci and lactobacilli
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19 141 populations showed a slight decline from day 15 onwards. The yeast and mould populations
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21 142 increased throughout ripening, approaching or surpassing (depending on the batch) 10^7 CFU/g
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23 143 of cheese. Because of their different morphology on the YGC plates, yeasts and moulds could
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25 144 be recorded separately (Table 1). Two *P. roqueforti* strains were distinguishable from one
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27 145 another owing to their different colored mycelia (PR3 dark green, PRG3 pale gray). Yeasts
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29 146 started at similar numbers in both batches, however, counts for batch 1 increased over those
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31 147 for batch 2 (maximum 6.1×10^7 and 5.75×10^5 CFU/g in batches 1 and batch 2 respectively).
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148 Variability in the cultivable microbial populations of dairy products is well known, even
149 when they are made from pasteurized milk [2, 19]. Different initial microbial loads (in
150 numbers and types) and post-pasteurization contamination may be responsible for the
151 majority of the differences observed in most studies.
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153 Microbial composition and dynamics as revealed by PCR-DGGE

154 Figures 1 and 2 show the composition and dynamics of the prokaryotic and eukaryotic
155 populations in batch 1 and batch 2 determined by PCR-DGGE. Panel A in both figures
156 corresponds to the DGGE patterns of batch 1, Panel B to those of batch 2. The profiles of the
157 cheese batches were relatively simple compared to the complex DGGE patterns of cheeses

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made of raw milk [4, 8, 16]. More bands were seen in the two milk samples, though most of them proved to be very faint. The intensity of an individual band is assumed to be a semi-quantitative measure of the abundance of the corresponding microorganism in the original population [14]. In addition to bands for *L. lactis* (band 4) and *Lactobacillus plantarum* (band 1), bands corresponding to *Weisella cibaria* (band 2) and *Actinobacterium* spp. (band 3) were found in the two milk samples (Line 1 in Figures 1A and 1B). As for the cheeses, three and four different bacterial bands were observed in batch 1 and 2 respectively. *L. lactis* was the most prominent band (band 4) in both. In the cheese samples, bands of lactococci were always accompanied by two bands identified as *Streptococcus thermophilus* (bands 5 and 6). In addition, in batch 2 another band located between bands 4 and 5 (band 7) was observed throughout manufacture and cheese ripening . This band was also present in the PCR-DGGE profile of the Flora Danica starter (Line S in Figure 1B). The sequence of this band was identical to that of *L. lactis* (band g). The DGGE pattern of Flora Danica produced two additional bands in the uppermost part of the gel (bands 8 and 9), the DNA sequence of which showed around 98 % similarity to *L. lactis* sequences. The presence of double bands corresponding to a single species may be due to heterogeneous copies of rRNA operons, a well-established limiting factor of the PCR-DGGE technique, but also to other artifacts related to the melting and re-association properties of related sequences [12, 17].

Figure 2 shows the DGGE profiles obtained with the primers for amplifying eukaryotic sequences. Five different bands were observed among the samples of the two batches. All were identified by isolation, reamplification, sequencing and comparison against sequences in databases. The sequences of two bands (band 1 and 2) were identical to those known for *Debaryomyces hansenii*; these were present in all samples of batch 1 after day 7 (except on day 15; line 5 in Figure 2A), while only a faint band was observed in the 15 day sample in

batch 2. In the lower part of the gels, three patent bands appeared in samples of both batches from day 7 onwards (bands 3, 4, and 5). The sequence of these bands matched those of *P. roqueforti*. As expected, PCR-DGGE analysis of Flora Danica with the eukaryotic primers gave no bands (Line S in Figure 2B).

The bands of both the prokaryotic and eukaryotic populations remained the same (qualitatively and quantitatively) during the entire cheese-making process. Of note is the absence in cheese of bands corresponding to bacteria from milk (except for that of *L. lactis*, a bacterium also present in the starter). Microorganisms in the pasteurized milk may be in a viable but non-cultivable state, and then do not progress into the cheese, or if they grow only small numbers are reached as compared to those obtained after the addition of starters. Although non declared, the *S. thermophilus* population detected may have come from the Flora Danica; as at least one of the two bands observed in the cheese samples was also detected for this undefined starter culture (the lowest band in line S in Figure 1B).

Comparison of plate count and PCR-DGGE results

The results obtained by culturing and DGGE showed similarities as well as patent differences. The heterogeneous distribution of microorganisms within the samples is a widely known cause of variation between and among culturing and DGGE results. The molecular method proved to be faster and cheaper in terms of running costs. Further, the analysis of the diversity and development of the microbial populations in the two batches over manufacturing and ripening (14 samples) was performed in a couple of weeks instead of the four months needed to finish conventional culturing analysis.

Intriguing is the differential results obtained for the lactobacilli with the two techniques. By conventional culturing, these were shown to form part of the majority populations,

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206 reaching numbers similar to those of lactococci (as in batch 2, day 60). However, although
207 bands related to lactobacilli were detected in milk, they were never detected in cheese.
208 Somehow, lactobacilli seem to be under-represented by the PCR-DGGE technique.
209 Lactobacilli are more resistant to lysozyme than other lactic acid bacteria species (and
210 particularly the lactococci), which may account for the differences. Species-specific primers,
211 such as those developed by [20], should be used for accurate tracking of lactobacilli.

212 Additionally, the unexpected population of *S. thermophilus* was not detected by
213 culturing; this bacterium is not declared by Chr. Hansen as a component of Flora Danica, and
214 was not isolated from it by Lodics and Steenson [13]. This technique is therefore preferable
215 for a rapid inspection of the diversity and development of the dominant populations during the
216 microbial typing of new products or processes. Once detected, culturing methods can be used
217 for the isolation, identification and typing of representative organisms. The use of selective
218 and differential media allowed the tracking of multiple microbial populations (nine microbial
219 groups), while only majority species could be followed by the PCR-DGGE technique (3 and 4
220 bands in batch 1 and 2, respectively). This is particularly important for populations used as
221 hygiene indicators (*S. aureus*, coliforms), which, because of their smaller numbers, might
222 only be detected by culturing. In addition, the different colony morphology shown by the two
223 *P. roqueforti* strains allowed their individual enumeration throughout ripening; this would not
224 be possible with the PCR-DGGE technique. On the other hand, counts cannot be directly
225 ascribed to a particular species, while this is possible with DGGE bands.

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227 **Conclusion**

228 Classic culturing and molecular methods have been repeatedly reported to provide
229 complementary results; this affirmation is strengthened by the results of the present work.

Both plate counting and PCR-DGGE analysis can be used for identifying and tracking majority populations throughout cheese manufacture and ripening, and both have their own advantages and drawbacks. They are both easy to perform, but the PCR-DGGE technique still requires dedicated equipment and reagents (thermocycler, nucleotides, polymerase, DGGE apparatus, etc.). Thus, the choice of using one or another ultimately depends on the purpose of the study if all necessary materials and instruments are available. However, the combination of the two undoubtedly improves the microbial characterization of the cheese-making process.

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293 **Table 1.-** Bacterial counts in log10 CFU mL or g determined in milk and cheese samples of batch
294 1 during cheesemaking and ripening.

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Microbial group (counting medium)	Stage of manufacture or ripening						
	Milk	Curd	3 day	7 day	15 day	30 day	60 day
Total aerobic counts (PCA)	5.01	8.53	9.13	9.28	8.54	8.41	8.58
Lactococci (M17)	5.80	8.41	9.11	9.20	8.58	8.37	8.57
Lactobacilli (MRS, pH 5.4)	3.03	7.26	8.37	8.49	8.18	7.74	7.48
Leuconostoc (MSE)	<1	<4.00	<4.00	<4.00	<4.00	<4.00	<4.00
Enterobacteriaceae (VRBG)	<1	3.88	3.89	2.40	<2.00	2.00	2.00
Coliforms (VRBL)	<1	4.13	3.90	2.30	<2.00	2.00	2.00
Enterococci (S-B)	<1	<2.00	<2.00	<2.00	<2.00	<2.00	5.67
Staphylococci (B-P)	1.48	2.18	2.74	2.00	<2.00	<2.00	2.30
Yeasts (YGC)	<1	2.00	<3.00	8.66	5.94	7.79	7.72
<i>P. roqueforti</i> PR3 (YGC)	<1	3.74	3.83	<4.00	<3.00	<4.00	4.30
<i>P. roqueforti</i> PRG3 (YGC)	<1	4.08	4.26	7.64	6.88	6.64	5.81

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297 The symbol < is used to indicate that numbers were lower than the detection limit.

Table 2.- Bacterial counts in log₁₀ CFU mL or g determined in milk and cheese samples of batch 2 during cheesemaking and ripening.

Microbial group (counting medium)	Stage of manufacture or ripening						
	Milk	Curd	3 day	7 day	15 day	30 day	60 day
Total aerobic counts (PCA)	4.60	7.46	9.13	9.25	9.09	8.95	8.60
Lactococci (M17)	3.91	7.75	9.35	9.35	9.36	9.02	8.48
Lactobacilli (MRS, pH 5.4)	<1	6.90	8.39	8.39	7.71	8.43	8.40
Leuconostoc (MSE)	<1	<4.00	<4.00	<4.00	<4.00	<4.00	<4.00
<i>Enterobacteriaceae</i> (VRBG)	<1	2.30	2.60	<2.00	<2.00	<2.00	<2.00
Coliforms (VRBL)	<1	2.30	2.78	<2.00	<2.00	<2.00	<2.00
Enterococci (S-B)	<1	<2.00	<2.00	<2.00	<2.00	<2.00	<2.00
Staphylococci (B-P)	<1	2.48	<2.00	2.00	<2.00	2.00	<2.00
Yeasts (YGC)	<1	2.00	<3.00	<3.00	5.76	5.00	<5.00
<i>P. roqueforti</i> PR3 (YGC)	<1	3.65	3.70	3.60	4.95	<5.00	<5.00
<i>P. roqueforti</i> PRG3 (YGC)	<1	3.93	4.18	4.11	6.74	6.96	6.53

The symbol < is used to indicate that numbers were lower than the detection limit.

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5 304 **Figure 1.-** DGGE profiles of microbial populations from cheese during manufacture and
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7 305 ripening, using amplicons of the V3 variable region of the bacterial 16S rRNA gene. Lanes
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9 306 Ma and Mb contain combined amplicons of identified strains used as controls; a) *Lactococcus*
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11 307 *garvieae*, b) *Lactobacillus plantarum*, c) *Leuconostoc mesenteroides*, d) *Streptococcus*
12
13 308 *parauberis*. e) *Enterococcus faecium*, f) *Enterococcus faecalis*, g) *Lactococcus lactis*, h)
14
15 309 *Escherichia coli*, i) *Lactobacillus paracasei*. Panel A contains samples from the first batch.
16
17 310 Sample 1: milk; 2: curd; 3, 4, 5, 6 and 7: cheese at 3, 7, 15, 30 and 60 days of ripening. Panel
18
19 311 B contains samples from the second batch (same order). Lane S shows the DGGE analysis of
20
21 312 a sample of the Flora Danica starter. Key to identified bands: 1) *L. plantarum*, 2) *Weissella*
22
23 313 *cibaria*, 3) *Actinobacterium* spp., 4) *L. lactis*, 5) and 6) *S. thermophilus*, 7) *L. lactis*, 8 and 9)
24
25 314 *L. lactis*-related sequences.

Figure 2.- DGGE profiles of moulds and yeasts using a PCR amplicon of the eukaryotic domain D1 of 26S rDNA. **Panel A** contains samples from the first batch. Sample 1: milk; 2: curd; 3, 4, 5, 6 and 7: cheese at 3, 7, 15, 30 and 60 days of ripening. **Panel B** contains samples from the second batch. Sample 1: milk; 2: curd; 3, 4, 5, 6 and 7: cheese at 3, 7, 15, 30 and 60 days of ripening. Lane S corresponds to the Flora Danica starter. Key to identified bands: 1) and 2) *Debaryomyces hansenii*, 3, 4, and 5) *Penicillium roqueforti*.