Assessment of microbial populations dynamics in a blue cheese by culturing and

denaturing gradient gel electrophoresis (DGGE)

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RUNNING TITLE: Culturing and DGGE cheese analysis

Key words:

cheese, blue cheese, microbial populations, cheese microbiology, culture-independent

methods, DGGE

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1 **Summary**

2

3   The composition and development of microbial population during the manufacture and

4 ripening of two batches of a blue-veined cheese was examined by culturing and polymerase

5 chain reaction (PCR) denaturing gradient gel electrophoresis (DGGE) (PCR-DGGE). Nine

6 selective and/or differential media were used to track the cultivable populations of total and

7 indicator microbial groups. For PCR-DGGE, the V3 hyper variable region of the bacterial

8 16S rRNA gene and the eukaryotic D1 domain of 28S rDNA were amplified with universal

9 primers, specific for prokaryotes and eukaryotes, respectively. Similarities and differences

10 between the results obtained by the culturing and the molecular method were recorded for

11 some populations. Culturing analysis allows minority microbial groups (coliforms,

12 staphylococci) to be monitored, although in this work PCR-DGGE identified a population of

13 *Streptococcus thermophilus* that went undetected by culturing. These results show that the

14 characterization of the microbial populations interacting and evolving during the

15 cheesemaking process is improved by combining culturing and molecular methods.

Introduction

16

17

18   The microbial characterization of dairy products has traditionally been performed using

19   culture dependent techniques employing non-selective, selective and differential media; this

20   allows the composition and development of majority and indicator populations present during

21   manufacturing and ripening to be monitored. However, a vast array of culture-independent

22   molecular methods is now being used for the microbial typing of food and food fermentations

23   [10, 11]. Molecular methods overcome culture-associated drawbacks such as the low

24   specificity of selective media and the inability to enumerate cells in a viable but non-

25   cultivable state. Most molecular methods rely on the amplification of rDNA or rRNA

26   sequences by the polymerase chain reaction (PCR) and the subsequent electrophoretic

27   analysis of the amplicons produced [1]. In addition, they are less laborious, faster and cheaper

28   than culture-based analyses. However, these techniques are not free from bias nor without

29   limitations [20] and are generally thought of as complementary to conventional methods,

30   providing a more precise microbial picture of food ecosystems.

31   Denaturing gradient gel electrophoresis (DGGE) and its relative, temperature gradient

32   gel electrophoresis (TGGE), were developed to analyse microbial communities based on

33   sequence-specific distinctions of 16S rRNA amplicons produced by PCR [15]. Separation is

34   based on the reduced electrophoretic mobility of partially melted double-stranded DNA

35   molecules in polyacrylamide gels with a linear gradient of denaturing agents (urea and

36   formamide) or temperature. A GC clamp of around 50 bp is attached to the 5'end of one of

37   the primers, preventing the two DNA strands from undergoing complete disassociation. If the

38   total DNA of a microbial community is used in PCR amplification these techniques can

39   provide the profile of the genetic diversity of the dominant populations. If total RNA is used

40 instead, the profiles reveal the metabolically-active populations [14]. Both PCR-DGGE and

41 PCR-TGGE have recently been used to study different microbial aspects of food-related

42 environments [7], including the diversity and dynamics of microorganisms present during

43 cheese manufacture and ripening [4, 5, 6, 8, 16].

44   The present paper reports the use of plate count methods and PCR-DGGE analysis for

45 studying microbial development, diversity and evolution during the manufacture and ripening

46 of a blue cheese made from pasteurized milk inoculated with commercial starters (mesophilic

47 lactic acid bacteria as acidifiers and *Penicillium roqueforti* spores as the ripening culture). The

48 aim of this work was to compare the results obtained with the culture-dependent and culture-

49 independent methods, assessing the advantages and drawbacks of each.

50

51 **Materials and Methods**

52

53 Cheese manufacture and sampling conditions

54   Two batches of blue-veined cheese were manufactured from pasteurized milk under

55 standard conditions [9]. Acidifying starters (Flora Danica) and ripening cultures (*Penicillium*

56 *roqueforti* strains PR3 and PRG3) were purchased from Chr. Hansen (Hørsholm, Denmark).

57 Milk, curd and cheese were sampled according to FIL-IDF standard 50B and transported to

58 the laboratory under refrigerated conditions. Culturing analyses were performed on the day of

59 sampling. For the isolation of DNA, milk, curd and cheese samples were stored at –20 ºC

60 until required.

61

62 Determination of microbial composition by plate counting

63　　　　Ten milliliters of milk or 10 g of curd and cheese were homogenized with 90 mL of a 2

64　% (w/v) sodium citrate solution at 45 ℃ in a Colworth Stomacher 400 (Seward Ltd., London,

65　UK) (for 3 x 1 min). Ten-fold serial dilutions were made in Maximum Recovery Diluent

66　(Scharlab, Barcelona, Spain) and plated in duplicate onto general and selective media, as

67　follows.

68　*Aerobic mesophilic counts*. Aerobic mesophilic bacteria were counted on Plate Count Agar

69　supplemented with 0.1 % skimmed milk (PCA; Merck, Darmstadt, Germany) after 72 h of

70　incubation in aerobiosis at 30 ℃.

71　Lactococci: Lactococci were grown on M17 agar (Scharlab) and enumerated after 48 h of

72　incubation at 30 ℃.

73　Lactobacilli. Lactobacilli were grown on de Man, Rogosa and Sharpe agar (MRS; Merck),

74　adjusted to pH=5.4 and enumerated after 72 h of incubation at 32 ℃ in a 5 % CO_2 enriched

75　incubator.

76　*Leuconostoc* spp. Dextran-producing leuconostocs were grown on Mayeux, Sandine and

77　Elliker agar (MSE; Biokar Diagnostics, Beauvais, France) and enumerated after five days of

78　incubation at 25 ℃.

79　Enterococci. Enterococci were grown on Slanetz and Bartley agar (S-B; Merck) and

80　enumerated after 24 h of incubation at 44 ℃.

81　Enterobacteria and coliforms. Enterobacteria and coliforms were grown on Violet Red Bile

82　Glucose agar (VRBG) and Violet Red Bile Lactose agar (VRBL) (both from Merck)

83　respectively, using the pour-plate and overlay technique. In brief, dilutions were mixed with

84　15 mL of agar and poured onto Petri dishes. After solidification, a second agar layer of 10 mL

85　was added. Bacteria were enumerated after 48 h of incubation at 30 ℃.

86   Staphylococci. Dilutions were grown on Baird-Parker agar (B-P; Merck) supplemented with

87   egg yolk tellurite solution (Merck). Black colonies with or without egg yolk clearing were

88   recorded after 24 h of incubation at 37 ºC.

89   Yeasts and moulds. Dilutions of milk, curd and cheese samples were plated on Yeast-Extract

90   Glucose Chloramphenicol agar (YGC; Merck). Yeasts and moulds were independently

91   recorded after 3-5 days of incubation at 25 ºC.

92   Except for the YGC plates, 100 μg/mL of cycloheximide (Merck) was added to all

93   enumeration media to inhibit the growth of moulds and yeasts.

94

95   PCR-DGGE analysis

96   Extraction of total microbial DNA.

97   Milk, cheese and starter samples homogenized in 2 % sodium citrate were used for the

98   isolation of total microbial DNA. DNA extraction was accomplished using a commercial kit

99   (QIAamp DNA Stool Mini Kit; Quiagen, GmbH, Hilden, Germany), following the supplier's

100   recommendations.

101

102   PCR amplification.

103   Total DNA was used as a template in PCR amplification of the V3 region of the bacterial 16S

104   rRNA gene using the universal primers F357 (5'-TACGGGAGGCAGCAG-3'), to which a 39

105   bp GC sequence was linked to give rise to GC-F357), and R518 (5'-

106   ATTACCGCGGCTGCTGG-3') [14]. The D1 domain of the 28S rRNA fungal gene was

107   amplified using primers GC-NL1 (5'-GCCATATCAATAAGCGGAGGAAAAG-3') and LS2

108   (5'-ATTCCCAAACAACTCGACTC-3') [3]. PCR was performed in a 50 μl volume using a

109   Taq-DNA polymerase master mix (75 mM Tris-HCl pH=8.5, 20 mM $(NH_4)_2S0_4$, 1.5 mM

110 MgCl$_2$, 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 ºC,

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).

121

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).

128

129 **Results and Discussion**

130

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

134   pasteurized milk, *Enterobacteriaceae*, coliforms, enterococci and staphylococci counts were

135   always in low numbers, though small variations between batches were recorded. No

136   *Staphylococcus aureus* strains were detected. Lactococci were found in the largest numbers;

137   in M17 the numbers recorded usually matched those of total bacterial aerobic counts in PCA,

138   reaching over 10^9 colony forming units (CFU)/g between day 3 and day 7 (Tables 1 and 2).

139   Lactobacilli grew from low numbers in milk up to 10^8 CFU/g by day 7, although their final

140   numbers were one log unit lower than those of lactococci. Both lactococci and lactobacilli

141   populations showed a slight decline from day 15 onwards. The yeast and mould populations

142   increased throughout ripening, approaching or surpassing (depending on the batch) 10^7 CFU/g

143   of cheese. Because of their different morphology on the YGC plates, yeasts and moulds could

144   be recorded separately (Table 1). Two *P. roqueforti* strains were distinguishable from one

145   another owing to their different colored mycelia (PR3 dark green, PRG3 pale gray). Yeasts

146   started at similar numbers in both batches, however, counts for batch 1 increased over those

147   for batch 2 (maximum 6.1 x 10^7 and 5.75 x 10^5 CFU/g in batches 1 and batch 2 respectively).

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.

152

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

158   made of raw milk [4, 8, 16]. More bands were seen in the two milk samples, though most of

159   them proved to be very faint. The intensity of an individual band is assumed to be a semi-

160   quantitative measure of the abundance of the corresponding microorganism in the original

161   population [14]. In addition to bands for *L. lactis* (band 4) and *Lactobacillus plantarum* (band

162   1), bands corresponding to *Weisella cibaria* (band 2) and *Actinobacterium* spp. (band 3) were

163   found in the two milk samples (Line 1 in Figures 1A and 1B). As for the cheeses, three and

164   four different bacterial bands were observed in batch 1 and 2 respectively. *L. lactis* was the

165   most prominent band (band 4) in both. In the cheese samples, bands of lactococci were always

166   accompanied by two bands identified as *Streptococcus thermophilus* (bands 5 and 6). In

167   addition, in batch 2 another band located between bands 4 and 5 (band 7) was observed

168   throughout manufacture and cheese ripening . This band was also present in the PCR-DGGE

169   profile of the Flora Danica starter (Line S in Figure 1B). The sequence of this band was

170   identical to that of *L. lactis* (band g). The DGGE pattern of Flora Danica produced two

171   additional bands in the uppermost part of the gel (bands 8 and 9), the DNA sequence of which

172   showed around 98 % similarity to *L. lactis* sequences. The presence of double bands

173   corresponding to a single species may be due to heterogeneous copies of rRNA operons, a

174   well-established limiting factor of the PCR-DGGE technique, but also to other artifacts

175   related to the melting and re-association properties of related sequences [12, 17].

176   Figure 2 shows the DGGE profiles obtained with the primers for amplifying eukaryotic

177   sequences. Five different bands were observed among the samples of the two batches. All

178   were identified by isolation, reamplification, sequencing and comparison against sequences in

179   databases. The sequences of two bands (band 1 and 2) were identical to those known for

180   *Debaryomyces hansenii*; these were present in all samples of batch 1 after day 7 (except on

181   day 15; line 5 in Figure 2A), while only a faint band was observed in the 15 day sample in

182   batch 2. In the lower part of the gels, three patent bands appeared in samples of both batches

183   from day 7 onwards (bands 3, 4, and 5). The sequence of these bands matched those of *P.*

184   *roqueforti*. As expected, PCR-DGGE analysis of Flora Danica with the eukaryotic primers

185   gave no bands (Line S in Figure 2B).

186   The bands of both the prokaryotic and eukaryotic populations remained the same

187   (qualitatively and quantitatively) during the entire cheese-making process. Of note is the

188   absence in cheese of bands corresponding to bacteria from milk (except for that of *L. lactis*, a

189   bacterium also present in the starter). Microorganisms in the pasteurized milk may be in a

190   viable but non-cultivable state, and then do not progress into the cheese, or if they grow only

191   small numbers are reached as compared to those obtained after the addition of starters.

192   Although non declared, the *S. thermophilus* population detected may have come from the

193   Flora Danica; as at least one of the two bands observed in the cheese samples was also

194   detected for this undefined starter culture (the lowest band in line S in Figure 1B).

195

196   Comparison of plate count and PCR-DGGE results

197   The results obtained by culturing and DGGE showed similarities as well as patent

198   differences. The heterogeneous distribution of microorganisms within the samples is a widely

199   known cause of variation between and among culturing and DGGE results. The molecular

200   method proved to be faster and cheaper in terms of running costs. Further, the analysis of the

201   diversity and development of the microbial populations in the two batches over manufacturing

202   and ripening (14 samples) was performed in a couple of weeks instead of the four months

203   needed to finish conventional culturing analysis.

204   Intriguing is the differential results obtained for the lactobacilli with the two techniques.

205   By conventional culturing, these were shown to form part of the majority populations,

206   reaching numbers similar to those of lacococci (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.

226

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.

230 Both plate counting and PCR-DGGE analysis can be used for identifying and tracking

231 majority populations throughout cheese manufacture and ripening, and both have their own

232 advantages and drawbacks. They are both easy to perform, but the PCR-DGGE technique still

233 requires dedicated equipment and reagents (thermocycler, nucleotides, polymerase, DGGE

234 apparatus, etc.). Thus, the choice of using one or another ultimately depends on the purpose of

235 the study if all necessary materials and instruments are available. However, the combination

236 of the two undoubtedly improves the microbial characterization of the cheese-making process.

237

242

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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.

295

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
***P. roqueforti* PR3 (YGC)**	<1	3.74	3.83	<4.00	<3.00	<4.00	4.30
***P. roqueforti* PRG3 (YGC)**	<1	4.08	4.26	7.64	6.88	6.64	5.81

296

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

298   **Table 2.-** Bacterial counts in log10 CFU mL or g determined in milk and cheese samples of batch 2

299   during cheesemaking and ripening.

300

| 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
***Enterobacteriaceae* (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
***P. roqueforti* PR3 (YGC)**	<1	3.65	3.70	3.60	4.95	<5.00	<5.00
***P. roqueforti* PRG3 (YGC)**	<1	3.93	4.18	4.11	6.74	6.96	6.53

301

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

303

304   **Figure 1.-** DGGE profiles of microbial populations from cheese during manufacture and

305   ripening, using amplicons of the V3 variable region of the bacterial 16S rRNA gene. Lanes

306   Ma and Mb contain combined amplicons of identified strains used as controls; a) *Lactococcus*

307   *garvieae,* b) *Lactobacillus plantarum,* c) *Leuconostoc mesenteroides,* d) *Streptococcus*

308   *parauberis.* e) *Enterococcus faecium,* f) *Enterococcus faecalis,* g) *Lactococcus lactis,* h)

309   *Escherichia coli,* i) *Lactobacillus paracasei.* Panel A contains samples from the first batch.

310   Sample 1: milk; 2: curd; 3, 4, 5, 6 and 7: cheese at 3, 7, 15, 30 and 60 days of ripening. Panel

311   B contains samples from the second batch (same order). Lane S shows the DGGE analysis of

312   a sample of the Flora Danica starter. Key to identified bands: 1) *L. plantarum*, 2) *Weissella*

313   *cibaria*, 3) *Actinobacterium* spp., 4) *L. lactis*, 5) and 6) *S. thermophilus*, 7) *L. lactis*, 8 and 9)

314   *L. lactis*-related sequences.
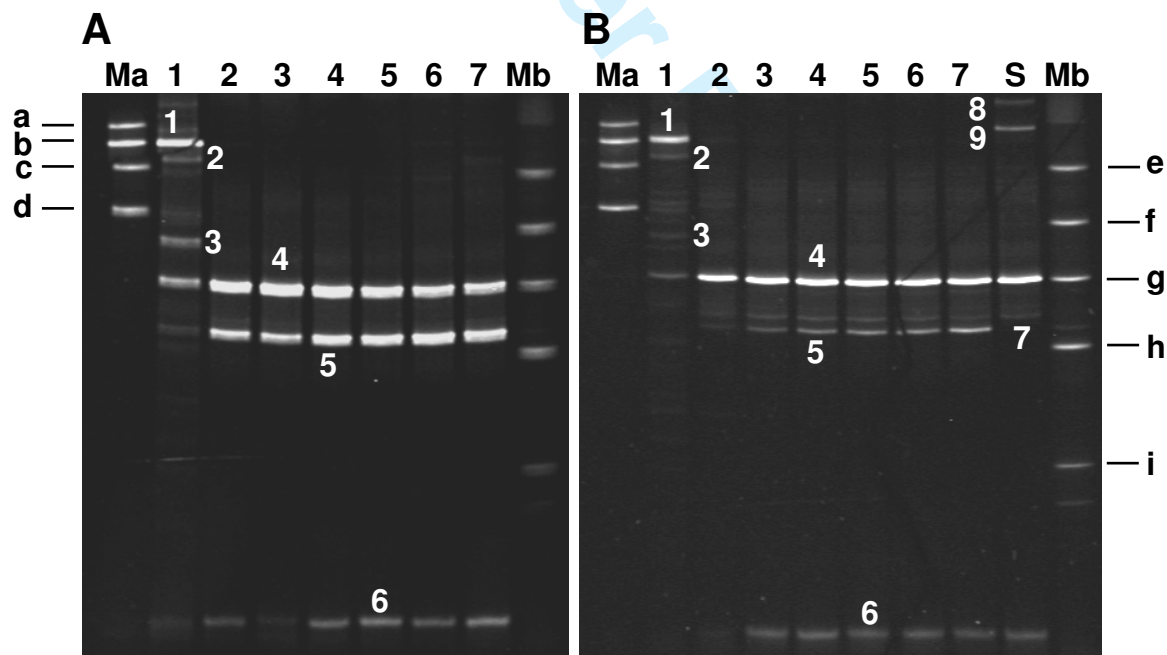
315   **Figure 2.-** DGGE profiles of moulds and yeasts using a PCR amplicon of the eukaryotic

316   domain D1 of 26S rDNA. **Panel A** contains samples from the first batch. Sample 1: milk; 2:

317   curd; 3, 4, 5, 6 and 7: cheese at 3, 7, 15, 30 and 60 days of ripening. **Panel B** contains samples

318   from the second batch. Sample 1: milk; 2: curd; 3, 4, 5, 6 and 7: cheese at 3, 7, 15, 30 and 60

319   days of ripening. Lane S corresponds to the Flora Danica starter. Key to identified bands: 1)

320   and 2) *Debaryomyces hansenii*, 3, 4, and 5) *Penicillium roqueforti*.

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