

Assessment of the microbial diversity at the surface of Livarot cheese using culture-dependent and independent approaches

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ABSTRACT

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The microbial diversity of the surface of a commercial red-smear cheese, *Livarot cheese*, sold on the retail market was studied using culture-dependent and independent approaches. Forty yeasts and 40 bacteria from the cheese surface were collected, dereplicated using single-strand conformation polymorphism (SSCP) analysis and identified using rRNA gene sequencing for the culture-dependent approach. The culture-independent approach involved cloning and sequencing of the 16S rRNA gene and SSCP analysis from total DNA extracted from the cheese. The most dominant bacteria were *Microbacterium gubbeenense*, *Leucobacter komagatae* and Gram-negative bacteria from the *Gamma-Proteobacteria* class. Fluorescence *in situ* hybridization (FISH) analysis was also used to study the cheese microbial diversity with class-level and specific rRNA-targeted probes for bacteria and yeasts, respectively. FISH analysis confirmed that *Gamma-Proteobacteria* were important microorganisms in this cheese. Four specific FISH probes targeting the dominant yeasts present in the cheese, *Candida catenulata*, *Candida intermedia*, *Geotrichum* spp. and *Yarrowia lipolytica*, were also designed and evaluated. These probes allowed the detection of these yeasts directly in cheese. The use of the rRNA gene-based approach combined with FISH analysis was useful to investigate the diversity of a surface microbial consortium from cheese.

Keywords: Smear cheese; yeast; coryneform bacteria; *Gamma-Proteobacteria*; fluorescence *in situ* hybridization.

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39 INTRODUCTION

40 Red-smear cheeses are economically important in many European countries. The
41 smear is a red-orange, often viscous, microbial mat that is characterised by a succession of
42 microbial communities, including yeasts and bacteria. During the first days of ripening, yeasts
43 colonise the cheese surface, use lactate and, in some cases, produce ammonia (Gori et al.
44 2007). These biological activities progressively lead to the deacidification of the cheese
45 surface, enabling the establishment of a bacterial community that is less acid-tolerant.

46 During the last few years, the microbial diversity of smear cheeses has been
47 investigated using both culture-dependent and culture-independent approaches such as rep-
48 PCR, FT-IR spectroscopy and 16S rRNA gene sequencing of culturable isolates (Mounier et
49 al. 2005, 2006), cloning and sequencing of the 16S rRNA gene and SSCP (Feurer et al. 2004a,
50 2004b), DGGE and TGGE (Ogier et al. 2004). These approaches have allowed a more
51 accurate characterisation of the surface microbiota of smear-ripened cheeses such as Munster,
52 Gubbeen and Limburger. The surface microbiota is dominated by yeasts, mainly
53 *Debaryomyces hansenii* and *Geotrichum candidum*, and Gram-positive catalase-positive
54 organisms such as coryneform bacteria and staphylococci (Maoz et al. 2003; Mounier et al.
55 2005; Feuerer et al. 2004; Goerges et al. 2008). These biodiversity studies have also revealed
56 the presence of subdominant populations of bacteria originally described in marine
57 environments such as Gram-negative *Halomonas* spp. and *Vibrio* spp., and Gram-positive
58 *Marinilactibacillus* spp. (Maoz et al. 2003; Mounier et al. 2005; Feuerer et al. 2004a, 2004b;
59 Ishikawa et al. 2007). Maoz et al. (2003) were the first to report these Gram-negative bacteria
60 in cheese and advanced the hypothesis that these bacteria might be indicative of hygienic

61 problems, *e.g.*, their prevalence might be due to poor growth of coryneform bacteria.
62 However, since the study of Maoz et al. (2003), these Gram-negative bacteria have been
63 isolated from other traditional European cheeses, including farmhouse and industrially-
64 produced cheeses (Mounier et al. 2005; Feurer et al. 2004a, 2004b; Rea et al. 2007; Ishikawa
65 et al. 2007). Therefore, they may be considered as components of the cheese surface
66 microbiota originating from the adventitious microbiota of the cheese-making environment.
67 Their abundance in cheese and their role during cheese ripening have thus to be evaluated.
68 Fluorescence *in situ* hybridization (FISH) is a culture-independent approach for the analysis
69 of complex microbial populations, for example in soil, sediments and activated sludge
70 (Amann et al., 1995). It has also been used for biodiversity studies in food samples such as
71 cheese (Ercolini et al. 2003a, 2003b; Cocolin et al. 2007), but these studies have mainly
72 focused on the ecology of lactic acid bacteria. To our knowledge, this technique has not yet
73 been applied for the characterisation of the yeasts and bacteria found at the surface of smear
74 cheeses.
75 The aim of this study was to describe the microbial diversity of a red-smear cheese using
76 culture-dependent and independent approaches. The yeast and bacterial diversity were studied
77 using **rRNA gene-based** approaches. Probes readily available in the literature or designed in
78 this study were then used to characterise *in situ* the yeasts and bacteria from the cheese
79 surface using FISH analysis.

80

81 **MATERIALS AND METHODS**

82 **Cheese sample.** The cheese studied was a commercial Livarot cheese sold on the retail
83 market. It is a soft surface-ripened cheese, similar to Munster, and made from pasteurised
84 milk. This cheese is generally ripened for at least three weeks at 8-12°C and subsequently
85 stored at 2-4°C for several weeks before being sold to the consumer.

86

87 **Microbiological analyses.** A piece of the cheese rind (depth: ~4 mm) was removed using a
88 sterile scalpel, and 2% trisodium citrate was added to yield a 1:10 dilution. The mixture was
89 homogenised with a mechanical blender (Ultra Turrax model T25, Labortechnik, Staufen,
90 Germany) at 8,000 rpm for 1 min. Part of this solution was kept for DNA extractions. The
91 bacteria and yeasts were enumerated on Brain Heart Infusion (BHI) Agar (Difco, Detroit, MI,
92 USA) supplemented with 44 mg/l amphotericine B and on Glucose Chloramphenicol Agar
93 (Merck-Eurolab, Fontenay-sous-Bois, France), respectively. The agar plates were incubated at
94 25°C for 3 d. Forty colonies of bacteria and 40 colonies of yeasts were selected at random
95 from countable plates and purified by restreaking twice on agar plates. They were stored at -
96 80°C in a 1:1 mixture of Brain Heart Infusion Broth (Difco)-Glycerol until characterisation.

97

98 **DNA extraction from cheese and from bacterial and yeast isolates.** For extracting DNA
99 from cheese, 1 ml of the cheese rind/trisodium citrate mixture was centrifuged for 10 min at
100 2700 x g at 4°C and resuspended in 1 ml of TE 10:1 (10 mmol/l Tris-HCl, 1 mmol/l EDTA,
101 pH 8.0). For bacteria and yeast isolates, biomass scraped from BHI Agar cultures that had
102 been incubated for 3 d was suspended in 1 ml of TE 10:1 and centrifuged for 10 min at 2700 x
103 g at 4°C. Cells were then maintained for at least 1h at -20°C. Total DNA was subsequently
104 extracted using the method of Gevers et al. (2001) with the following modification. The lysis
105 buffer contained 1330 U ml⁻¹ lysostaphin and 40 mg ml⁻¹ lysozyme for bacterial isolates, 1330
106 U ml⁻¹ lyticase for yeast isolates and 1330 U ml⁻¹ lysostaphin, 40 mg ml⁻¹ lysozyme and 1330
107 U ml⁻¹ lyticase for cheese samples.

108

109 **SSCP analysis.** The bacterial and yeast community compositions of cheese and the yeast and
110 bacterial isolates were assessed by PCR-single-strand conformation polymorphism (SSCP)

111 analyses. Primers w34 (5'-TTACCGCGGCGTGCTGGCAC-3') and w49 (5'-
112 ACGGTCCAGACTCCTACGGG-3') were used to amplify the V3 variable regions of the
113 bacterial 16S rRNA genes (Duthoit et al. 2003), and primers NL3A (5'-
114 GAGACCGATAGCGAACAAAG-3') and NL4 (5'-GGTCCGTGTTTCAAGACGG-3') were
115 used to amplify the 5' end of the yeast 26S rRNA genes (Voigt et al. 1999). SSCP analyses
116 were performed as previously described by Feurer et al. (2004a). Following SSCP analysis of
117 yeast and bacteria isolates, the D1/D2 region of the large-subunit rRNA gene and the
118 complete 16S rRNA gene of one to five isolates that had similar SSCP patterns were
119 sequenced as described in the following section. This library of SSCP patterns was used to
120 identify the peaks of the SSCP patterns of whole bacterial and yeast communities in cheese.

121

122 **Sequencing of rRNA genes from bacterial and yeast isolates.** Yeast isolates were identified
123 by sequencing of the D1/D2 region of the 26 rRNA gene as previously described (Kurtzman
124 and Robnett 1997, 1998). The 16S rRNA gene of bacterial isolates from cheese was amplified
125 using primers pA (5'-AGAGTTTGATCCTGGCTCAG-3') and pH (5'-
126 AAGGAGGTGATCCAGCCGCA-3'), as previously described (Edwards et al. 1989). The
127 resulting amplicons were sequenced by Cogenics (Meylan, France), using the pA and pH
128 primers. The sequences were then assembled using the CAP3 programme (Huang 1996) and
129 compared to the GenBank database using the Basic Local Alignment Search Tool (BLAST)
130 (<http://www.ncbi.nlm.nih.gov/BLAST/>) to determine the closest known relatives of the 16S
131 rRNA gene or 26S rRNA gene sequences.

132

133 **Cloning and sequencing of 16S rRNA gene amplified from cheese DNA sample.** One
134 microlitre of DNA sample extracted from cheese was amplified using primers pA and pH as
135 previously described (Edwards et al. 1989). The PCR product was ligated into the pCR4-

136 TOPO vector (Invitrogen, Carlsbad, CA). Recombinant pCR4-TOPO plasmids were used to
137 transform *E. coli* TOP10 One Shot chemically competent cells according to manufacturer's
138 instructions (Invitrogen). Ninety transformant clones were randomly picked, and inserts were
139 amplified by PCR using M13 primers (Messing 1983). Seven microlitres of PCR products
140 were digested with 5 U of the *HaeIII* restriction enzyme overnight at 37°C. The restriction
141 fragments were analysed by electrophoresis on a 2.5 % (w/v) agarose gel [1.66% w/v
142 SeaKem-GTG agarose (FMC BioProducts, Rockland, ME, USA), and 0.84% w/v standard
143 agarose] in 0.5x TBE buffer at 110 V for 2 h. The restriction fragments were visualised on a
144 UV transilluminator after ethidium bromide staining. After photography using a digital
145 camera, the resulting fingerprints were analysed. The rRNA gene insert of each clone
146 representative showing distinct restriction profile was amplified using T3 and T7 primers as
147 described by the manufacturer. The resulting amplicons were sequenced by Cogenics, using
148 primers T3 and T7.

149

150 **Phylogenetic analyses.** Phylogenetic analyses were conducted using MEGA4 software
151 (Tamura et al. 2007).

152

153 **FISH analysis**

154 FISH probes. The list of oligonucleotide probes labelled with Cy3, fluorescein or rhodamine
155 (Eurogentec, Seraing, Belgium) is shown in Table 1. . Four of the probes (Ccat, Cint, Geo and
156 Ylip) were designed in order to visualise the different yeast species present in this cheese as
157 described in the following section. Details on oligonucleotide probes used in this study are
158 available at probeBase (www.microbial-ecology/probebase/) (Loy et al. 2007). The absence
159 of significant autofluorescence of cells and unspecific adhesion of probes in cheese samples
160 was checked by performing FISH experiments with a nonsense probe labelled with either Cy3,

161 fluorescein or rhodamine.

162

163 Design and evaluation of FISH probes. Specific oligonucleotide probes to visualise *in situ*
164 *Candida catenulata*, *Candida intermedia*, *Geotrichum* spp. and *Yarrowia lipolytica* were
165 designed using Primrose software (<http://www.bioinformatics-toolkit.org/index2.html>). A
166 dataset comprising 26S rRNA gene sequences of 13 yeast species commonly found in cheese
167 (at least three sequences from each yeast species), *C. intermedia*, *C. catenulata*, *C. rugosa*, *C.*
168 *zeylanoides*, *Cryptococcus laurentii*, *Debaryomyces hansenii*, *Kluyveromyces marxianus*,
169 *Pichia membranifaciens*, *Saccharomyces dairenensis*, *Torulaspora delbrueckii*, *Trichosporon*
170 *ovoides*, *Y. lipolytica* and *G. candidum*, and the 26S rRNA gene sequences of the yeasts
171 isolated from this cheese, was used to design specific FISH probes. Each probe had at least
172 three mismatches with the rRNA of non-target yeasts, except the *Geotrichum* spp. probe,
173 which had two mismatches with the *C. catenulata* 26S rRNA gene. The appropriate
174 stringency of hybridization was determined by a series of *in situ* hybridizations performed at
175 formamide concentrations of 0, 10, 20, 25, 30, 35, 40, 45, 55 and 70% at 46°C. For each
176 formamide concentration, the fluorescence intensity of cells was evaluated for the target
177 organism and a non-target organism and compared using the DAIME image analysis software
178 (Daims et al. 2006). *C. intermedia*, *Y. lipolytica*, *C. catenulata* and *C. catenulata* were the
179 non-target organisms used for Ccat, Cint, Geo and Ylip probes, respectively. The formamide
180 concentration that conferred the maximal fluorescence intensity to the target organism
181 compared with the non-target organism was used for further FISH detection in cheese.

182

183 Fixation of cheese samples for FISH analysis. Cheese rind (2.4 g) was mixed with 10 ml of
184 2% trisodium citrate and homogenised with a mechanical blender (Ultra Turrax model T25,
185 Labortechnik, Staufen, Germany) at 8,000 rpm for 1 min. This mixture was centrifuged and
186 resuspended in 1 ml of 1x PBS (10 mmol/l sodium phosphate buffer, 130 mmol/l NaCl, pH

187 7.2). The sample was then fixed using the PFA-fixation procedure. Five hundred microlitres
188 of the cheese rind mixture were mixed with 1.5 ml of ice-cold 4% paraformaldehyde in 1x
189 PBS and incubated at 4°C for 4 h. The fixed sample was then centrifuged for 5 min at 15,000
190 x g at 4°C and washed three times using 1 ml of 1x PBS. The sample was resuspended in 500
191 µl of 96% ice-cold ethanol and 500 µl of 1x PBS. After fixation, PFA-fixed samples were
192 maintained at -20°C until FISH analysis.

193

194 Fluorescence *in situ* hybridization. Except for relative quantification of cells, fluorescence *in*
195 *situ* hybridization was performed as previously described (Bond et al. 1999). The only
196 difference was that the hybridization step was performed for 3 h. Cells were visualised using
197 an epifluorescence microscope (Olympus System Microscope Model BX51) equipped with an
198 RS Photometrics CoolSNAP camera. The microscope was equipped with a U-MWB2
199 fluorescence mirror unit (460–490 nm band-pass excitation filter, 500 nm dichroic mirror and
200 520 nm long-pass emission filter) for FITC fluorochrome, and with a U-MWG2 fluorescence
201 mirror unit (510–550 nm band-pass excitation filter, 570 nm dichroic mirror and 590 nm
202 long-pass emission filter) for Cy3 and rhodamine fluorochromes.

203 Dual staining with the *Gamma-Proteobacteria* probe Gam42a or *Actinobacteria* probe
204 HGC69a in combination with the bacteria-specific probe, EUB338, was performed to
205 quantify the relative amount of *Gamma-Proteobacteria* and *Actinobacteria*. The hybridization
206 step was performed as described above except that the microscope slide was dipped into 0.5%
207 agarose prior to dehydration of the sample, and excess agarose was removed from both sides
208 of the slide after solidification on a cold Petri-dish plate. Fifteen countable optical fields were
209 randomly chosen. The number of cells labelled by the Gam42a or HGC69a probes compared
210 with the number of cells stained by the EUB338 probe was determined manually. For
211 quantification using the HGC69a probe, an enzymatic treatment with 70,000 units of

212 lysozyme in TE 10/1 for 5 min at 37°C, was applied to the fixed sample prior to hybridization.
213 This enzymatic treatment was optimised by varying the treatment time with lysozyme using
214 PFA-fixed pure cultures of *Microbacterium gubbeenense*, *Leucobacter* sp., *Arthrobacter*
215 *arilaitensis* and *Staphylococcus xylosus* grown to the log and stationary phase. This enzymatic
216 treatment was checked to make sure that this procedure did not lead to the lysis of the other
217 bacterial species *e.g.*, Gram-negative bacteria present in the cheese sample.

218

219 RESULTS

220 *Bacterial diversity.* Bacterial cell count on the cheese surface after cultivation on BHI agar
221 was 3.7×10^8 CFU/g. The bacteria from the surface of the Livarot cheese could be divided
222 into two groups. The first group was composed of Gram-positive catalase-positive bacteria
223 from the *Micrococcineae* suborder, and the second one of bacteria from the *Gamma-*
224 *Proteobacteria* class. Eight different taxons were identified using both cloning/sequencing
225 and culture-dependent analysis (Table 2). Three bacteria were identified using both
226 techniques: *Microbacterium gubbeenense*, *Leucobacter komagatae* and *Halomonas* sp. while
227 *Arthrobacter arilaitensis*, *Lactococcus lactis* and *Pseudoalteromonas* sp. were only identified
228 using cloning/sequencing, and *Vibrio littoralis* and *Hafnia alvei* were only identified using
229 culture-dependent analysis. *M. gubbeenense* was the most abundant, followed by *Leucobacter*
230 *komagatae* and *Halomonas* sp. using cloning/sequencing (Figure 1A), while *Halomonas* sp.
231 was the most abundant, followed by *Leucobacter komagatae* and *M. gubbeenense* in the
232 culture-dependent analysis (Figure 1B). SSCP analysis made it possible to identify the same
233 taxons as for the culture-dependent techniques since it is based on SSCP pattern library of
234 culturable isolates (Figure 1C).

235

236 *Yeast diversity.* Yeast cell count on the cheese surface was 2.4×10^6 CFU/g. Five yeast

237 species, *C. catenulata*, *C. intermedia*, *Geotrichum candidum*, *Geotrichum* sp. and *Yarrowia*
238 *lipolytica*, were identified using culture-dependent analysis (Table 3) and SSCP analysis (data
239 not shown). The isolates identified as *G. candidum* and *Geotrichum* sp. had two distinct
240 colony morphotypes as well as two different SSCP patterns (data not shown). Culture-
241 dependent analysis showed that *C. catenulata* with 22 of 40 isolates (55%) followed by
242 *Geotrichum* spp. with 12 of 40 isolates (30%) dominated the yeast flora.

243

244 *FISH analyses.* The four specific FISH probes designed in this study allowed the direct
245 visualisation of *C. catenulata*, *C. intermedia*, *Geotrichum* sp. and *Y. lipolytica* in pure cultures
246 and in cheese as shown in Figure 2. Fluorescence intensities were lower in cheese than in pure
247 cultures, possibly because the rRNA content of yeast cells obtained from cheese was lower
248 than in cells obtained from liquid cultures. **Probe accessibility may also be reduced in cheese**
249 **samples.** *Geotrichum* spp. cells had a slightly higher autofluorescence than the other yeast
250 species. However, *Geotrichum* spp. were easily distinguishable from other yeast species
251 because of their recognisable cell morphology. Using FISH analysis, it was also found that *C.*
252 *catenulata* followed by *Geotrichum* sp. (data not shown) were the most abundant species, as
253 observed for the culture-dependent analysis.

254 **Figure 3A shows an image of FISH analysis using EUB338 and EUK516 probes.** The
255 importance of *Gamma-Proteobacteria* in this cheese observed using cloning/sequencing and
256 culture-dependent analyses was confirmed using FISH analyses (Figures 3B and C). It is
257 interesting to note that there were differences in the relative quantity of *Gamma-*
258 *Proteobacteria* and *Micrococcineae* determined by cloning/sequencing, culture-dependent
259 and FISH analyses (Table 4). FISH analysis using Gam42a and HGC69a probes actually
260 showed that *Gamma-Proteobacteria* and *Actinobacteria* represented $91.3 \pm 5\%$ and $19.6 \pm$
261 6% , respectively. In contrast, culture-dependent analyses resulted in the identification of an

262 equal number of isolates from the *Gamma-Proteobacteria* class and *Micrococcineae* suborder,
263 and cloning/sequencing resulted in the identification of 75% *Micrococcineae* and 21.7%
264 *Gamma-Proteobacteria* clones.

265 **DISCUSSION**

266 During the ripening of smear cheeses, the bacterial surface microbiota is generally
267 dominated by Gram-positive catalase-positive bacteria from the actinomycetes and the
268 clostridial branches: *Corynebacterium* spp., *Arthrobacter* spp., *Microbacterium* spp. and
269 *Staphylococcus* spp. (Bockelmann et al. 1997; Feurer et al. 2004a, b; Mounier et al. 2005; Rea
270 et al. 2007; Goerges et al. 2008). Three Gram-positive bacteria from the *Micrococcineae*
271 suborder were identified in this cheese. *A. arilaitensis* was subdominant while *L. komagatae*
272 and *M. gubbeenense* were dominant in this cheese. *A. arilaitensis* and *M. gubbeenense* have
273 been previously reported in other types of European smear cheeses (Brennan et al. 2002;
274 Irlinger et al. 2005; Goerges et al. 2008). *Leucobacter komagatae*, described for the first time
275 by Takeuchi et al. (1996), was found in this cheese and to our knowledge, this species has not
276 been reported in other smear cheeses.

277 Gram-negative bacteria are generally subdominant on the surface of smear cheeses
278 (Mounier et al. 2005; Feurer et al. 2004a, 2004b; Rea et al. 2007). Four genera of Gram-
279 negative bacteria, *Vibrio* sp., *Halomonas* sp., *Pseudoalteromonas* sp. and *Hafnia* sp. were
280 identified in this study. These bacteria have already been found in other smear cheeses
281 (Mounier et al. 2005; Feurer et al. 2004a; Ogier et al. 2004). This is not the first time that
282 *Halomonas* spp. are part of the dominant microbiota in smear cheeses. Indeed, Mounier et al.
283 (2005) found that *H. venusta* represented 22 isolates of 50 (44 %) made from an Irish
284 farmhouse cheese at a mid-stage of ripening and Bockelmann et al. (2003) found that 35 % of
285 the isolates made from semi-hard smear cheeses were *Halomonas* sp.. The occurrence of these
286 Gram-negative bacteria is not surprising in smear cheeses because these bacteria are salt-

287 tolerant, grow at pHs occurring during cheese ripening (pH from 5-7) and are able to grow at
288 temperatures encountered during ripening (10-16°C). These Gram-negative bacteria also have
289 the ability to grow at low temperatures (4-6°C) in contrast with coryneform bacteria that
290 rarely grow below 10°C (Ridell and Korkeala 1997; Ventosa et al. 1998; Masoud and
291 Jakobsen 2005). Therefore, these Gram-negative bacteria may grow during storage at 4°C.
292 Their growth may also be promoted if the cheese is stored at an inadequate temperature
293 between the time of cheese manufacture and sale at the retail market. It would be necessary to
294 follow *Gamma-Proteobacteria* dynamics during ripening and subsequent storage to validate
295 these hypotheses.

296 In this study, the bacterial and yeast diversity at the surface of Livarot cheese surface
297 was investigated using both culture-dependent and culture-independent techniques. This is not
298 the first time that such techniques have been combined to study smear cheese diversity.
299 Feurer et al. (2004a) showed that the combination of these techniques provides an accurate
300 view of the cheese surface bacterial diversity in a French red-smear cheese. The
301 complementarity of these techniques was also demonstrated in the present study. For example,
302 *H. alvei* and *V. litoralis* were only identified using the spread-plate technique, while
303 *Pseudoalteromonas* sp. and *A. arilaitensis* were only identified using cloning and sequencing
304 of the 16S rRNA gene.

305 In this study, there were differences in the estimation of the relative quantities of
306 *Gamma-Proteobacteria* and *Micrococcineae* depending on the techniques used to investigate
307 the bacterial microbiota. These differences may be due to the primer sets used for the cloning
308 and sequencing strategy, which may have led to preferential amplification of *Actinobacteria*,
309 for example, of bacteria from the *Micrococcineae* suborder (Feurer et al. 2004a). The
310 universal primers pA and pH did not match perfectly the 16S rRNA gene of *V. litoralis* and *H.*
311 *alvei* which were only identified using SSCP analysis and the culture-dependent approach.

312 This could explain why these two species were not detected using the cloning and sequencing
313 strategy. Using these universal primers, the absence of amplification of the 16S rRNA gene
314 has already been found for other bacteria from cheese (Feurer et al. 2004a). DNA from dead
315 cells may also have been amplified. Moreover, in this study, the isolation of bacterial clones
316 was only performed on one medium that contained 0.5% NaCl, whereas for example, optimal
317 NaCl concentration for growth of moderately halophilic bacteria is in the range of 3.5-8%
318 NaCl (Ventosa et al. 1998). The use of media with higher NaCl content (3.5 and 7% NaCl)
319 might result in the isolation and identification of many more bacterial species related to
320 moderately halophilic bacteria as previously shown by Ishikawa et al. (2007). FISH analysis
321 may also lead to biased results regarding the relative quantities of *Gamma-Proteobacteria* and
322 *Actinobacteria* because of an unequal permeation of Gram-positive and Gram-negative cells.
323 The permeation of Gram-positive and Gram-negative bacteria was only optimized using cells
324 obtained from liquid cultures and permeation properties may differ in cells obtained from
325 cheese. A proteinase K treatment may also have been useful to optimize the permeabilization
326 of Gram-positive bacteria in the presence of Gram-negative bacteria as previously reported by
327 Ercolini et al. (2006). Therefore, it cannot be concluded based on FISH analysis whether
328 *Gamma-Proteobacteria* are the most dominant bacteria in this cheese.

329 *C. catenulata* followed by *Geotrichum* spp., *C. intermedia* and *Y. lipolytica* were
330 found to dominate the yeast community. The same species, except for *C. intermedia*, were
331 reported by Larpin et al. (2006) on Livarot cheese at a late stage of ripening, e.g., 60 days
332 after cheese-making, which would be a cheese “age” similar to that of the cheese sampled in
333 this study. Larpin et al. (2006) also developed a real-time PCR assay to quantify four
334 important yeast species in Livarot cheese. This molecular approach proved to be useful for
335 detecting and enumerating these yeast species directly in cheese. However, it appeared that
336 DNA extraction yields from cheese were low, with extraction yields varying between 0.4%

337 and 10.5% (Larpin et al. 2006). In the present study, four specific FISH probes for *C.*
338 *catenulata*, *Geotrichum* spp., *C. intermedia* and *Y. lipolytica* were designed, evaluated both in
339 pure cultures and in a cheese sample, and were found to be efficient in detecting these four
340 yeast species. To our knowledge, these probes are the first probes described for detecting
341 yeasts that are common in cheese. These probes may be used for absolute quantification or for
342 the study of yeast diversity in cheese.

343 Only one cheese was sampled in this study and it raises the question of whether the
344 yeast and bacterial species identified are representative of the microbiota of Livarot cheese.
345 They are likely to be representative since the same species of yeasts were found in another
346 batch of the same cheese (Larpin et al. 2006) and since it has been shown in different
347 European smear cheeses that their bacterial communities were stable over time (Maoz et al.
348 2003, Feurer et al. 2004b, Rea et al. 2007). However, further studies on other cheese samples
349 are required to confirm the microbial structure of this cheese.

350 In conclusion, the use of FISH as a complementary technique of the rRNA gene-based
351 approach may be an effective tool to characterise the microbial diversity of red-smear cheeses.
352 This technique will undoubtedly provide new insights into the understanding of microbial
353 communities in cheese.

354

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357

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476 **Table 1.** List of FISH probes used in this study.

477

Probe	Specificity	Sequence (5'-3') of the probe	Target site (rRNA position) ^a	% FA ^b	References
Bacteria					
S-D-Bact-0338-a-A-18 (Eub338)	Most Eubacteria	GCTGCCTCCCGTAGGAGT	16S (338-355)	20, 25 or 35 ^c	Amann et al. (1990)
L-C-gProt-1027-a-A-17 (Gam42a)	<i>Gamma-Proteobacteria</i>	GCCTTCCCACATCGTTT	23S (1027-1043)	35	Manz et al. (1992)
L-P-Grps-1901-a-A-18 (HGC69a)	<i>Actinobacteria</i>	TATAGTTACCACCGCCGT	23S (1901-1918)	25	Roller et al. (1994)
Yeast					
EUK516	Eukarya	ACCAGACTTGCCCTCC	18S (502-517)	20	Amann et al. (1990)
Ccat	<i>Candida catenulata</i>	TTTATCTCCCGCGCCT	26S (612-627)	20	This study
Cint	<i>Candida intermedia</i>	TTATCCACCCCTAGCA	26S (1415-1430)	20	This study
Geo	<i>Geotrichum</i> sp.	TTACGGGGCTGTCACCCT	26S (324-341)	20	This study
Ylip	<i>Yarrowia lipolytica</i>	CACTCATTTCCTTCCC	26S (2729-2744)	20	This study

478 ^a *Escherichia coli* rRNA numbering (Brosius et al. 1981)

479 ^b Formamide concentration for optimum probe stringency

480 ^c Formamide concentration when used in combination with EUK516, HGC69a and Gam42a probes, respectively.

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Table 2. Identification of the bacteria from the surface of Livarot cheese.

Strains	Accession number	Closest phylogenetic affiliation in the GenBank/EMBL/DDBJ/PDB databases (Accession number)	% similarity
CC1 ^a	FJ394918	<i>Halomonas</i> sp. (AJ640133)	99
CC2 ^a	FJ394919	<i>Microbacterium gubbeenense</i> (EU863414)	100
CC4 ^b	FJ394925	<i>Vibrio littoralis</i> (DQ097524)	99
CC16 ^b	FJ394920	<i>Hafnia alvei</i> (FM179942)	99
CC20 ^a	FJ394921	<i>Leucobacter komagatae</i> (EU370411)	99
GC4 ^c	FJ394922	<i>Lactococcus lactis</i> (AE006456)	99
GC27 ^c	FJ394923	<i>Pseudoalteromonas</i> sp. (EU365474)	99
GC54 ^c	FJ394924	<i>Arthrobacter arilaitensis</i> (AJ609626)	99

484 ^aStrains identified using the culture-dependent approach and cloning and sequencing of the 16S rRNA gene.

485 ^bStrains only identified in the culture-dependent approach

486 ^cStrains only identified using cloning and sequencing of the 16S rRNA gene.

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500 **Table 3.** Identification of the yeasts from the surface of Livarot cheese.

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Isolate	Closest phylogenetic affiliation in the GenBank/EMBL/DDBJ/PDB databases	Accession no.	Similarity (%)	References	% of the total culturable yeast microbiota
Y5	<i>Candida intermedia</i>	AJ508588	100	Daniel and Meyer (2003)	7.5
Y7	<i>Yarrowia lipolytica</i>	AJ508570	100	Daniel and Meyer (2003)	7.5
Y9	<i>Candida catenulata</i>	U45714	100	Kurtzman and Robnett (1997)	55
Y10	<i>Geotrichum</i> sp.	AB294537	99	Suzuki M. and Kanayama K. (unpublished)	22.5
Y35	<i>Geotrichum candidum</i>	EU194453	99	Nielsen, D. S., Jacobsen, T., Jespersen, L., Koch, A. G. and Arneborg, N. (unpublished)	7.5

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504 **Table 4.** Comparison of the relative quantity of *Actinobacteria* and *Gamma-Proteobacteria* in
 505 Livarot cheese using cloning/sequencing, culture-dependent and FISH analyses.

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Percentage of population as a function of the technique used			
Class/suborder	Cloning/sequencing	Culture-dependent	FISH
<i>Micrococcineae</i>	75	50	19.6 (13.6-25.6) ^a
<i>Gamma-Proteobacteria</i>	21.7	50	91.3 (86.3-96.3)

507 ^a 95% confidence interval

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510 **Figure legends.**

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512 Figure 1. Taxonomic grouping of Livarot cheese gene clones (A) and culturable isolates (B)
513 of bacteria based on 16S rRNA gene sequences. (C) Single-strand conformation
514 polymorphism analysis of the cheese surface bacterial community (a, *Microbacterium*
515 *gubbeenense*; b, *Leucobacter komagatae*; c, *Hafnia alvei*; d, *Halomonas* sp.; e, *Vibrio littoralis*;
516 f, *Lactococcus lactis*; * non-assigned peak).

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518 Figure 2. FISH analysis of *Candida catenulata* (A), *Geotrichum* sp. (B), *Candida intermedia*
519 (C) and *Yarrowia lipolytica* (D) using EUK516 probe (A and B, green; C and D, red) and
520 specific probes (A and B, red; C and D, green) in pure cultures (magnification x1000) and in
521 Livarot cheese (magnification x400).

522

523 Figure 3. Combined epifluorescence micrographs of yeasts and total bacteria labelled with
524 EUK516 (red) and EUB338 (green) probes (A) and an optical field showing total bacteria (B)
525 labelled with EUB338 probe and *Gamma-Proteobacteria* (C) labelled with Gam42a probe in
526 Livarot cheese.

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530 **Figure 1.**

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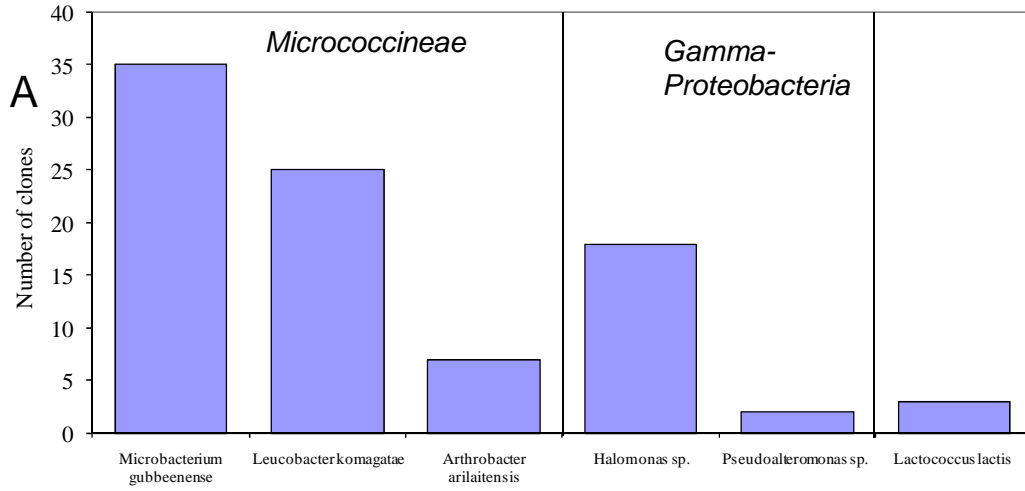
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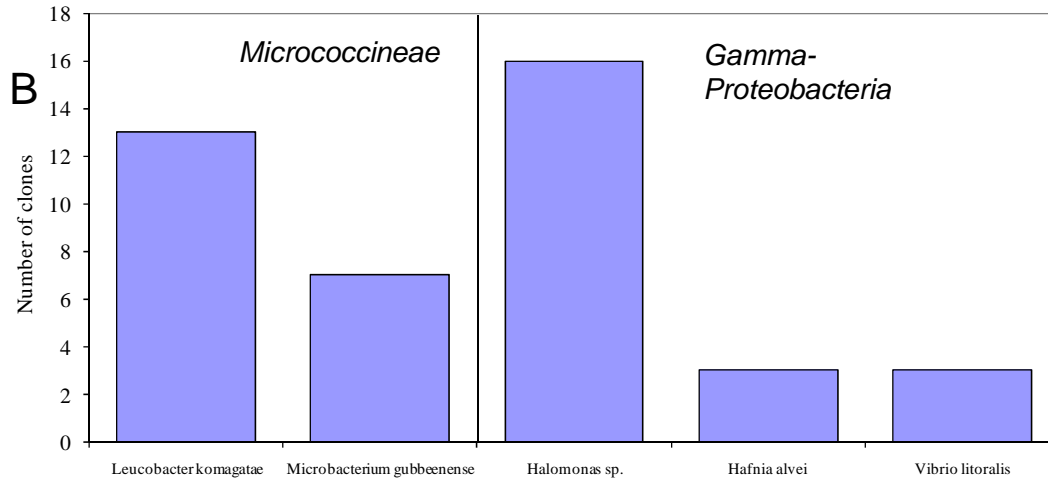
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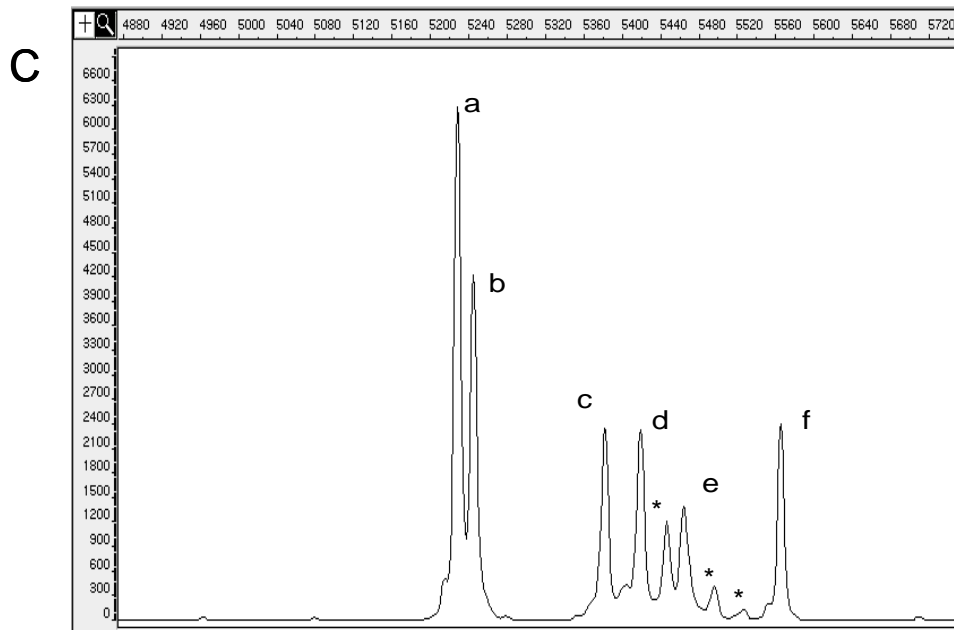
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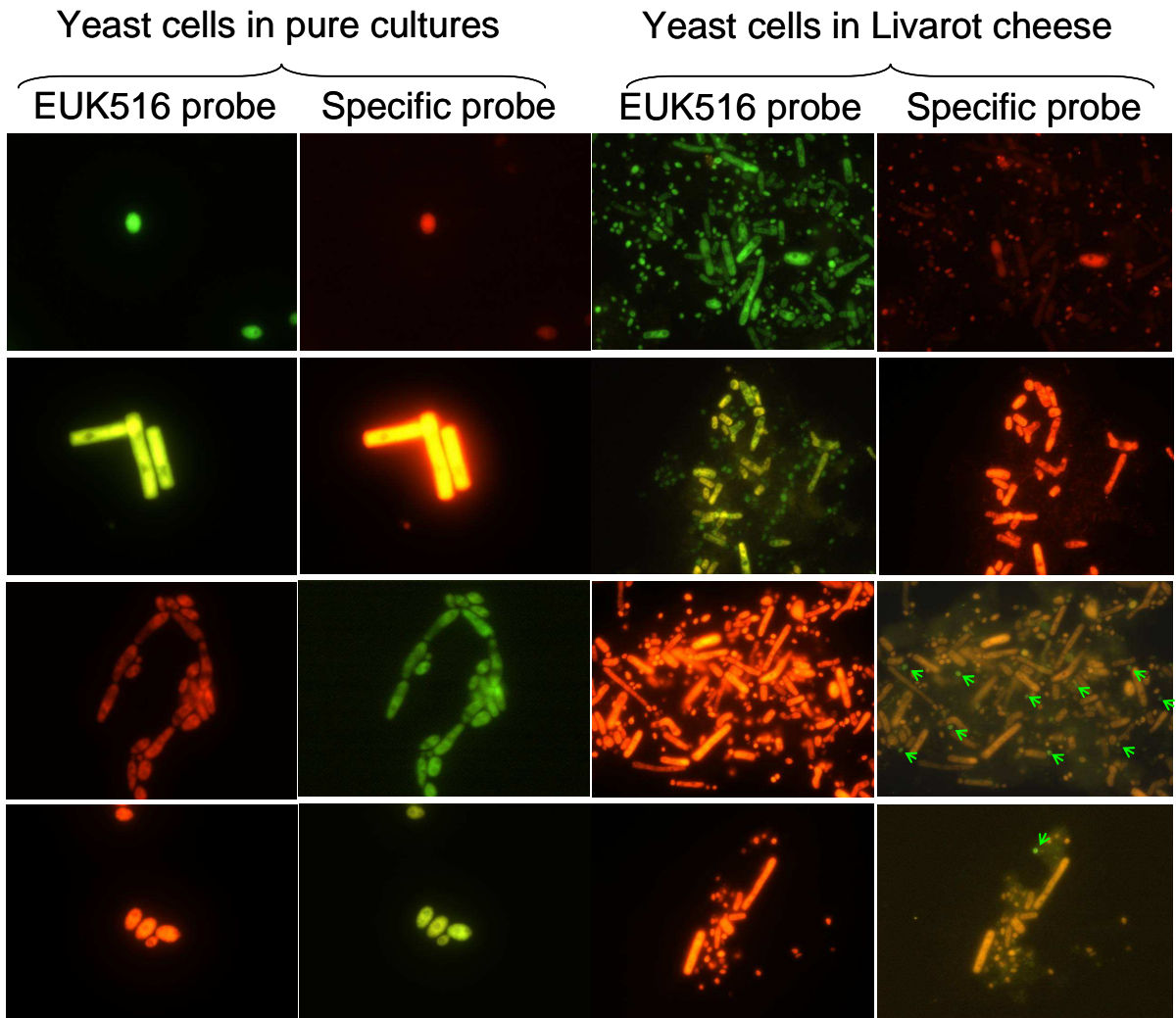
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Figure 2.



603 **Figure 3.**

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