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ABSTRACT

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. Fourty 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.

INTRODUCTION

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

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

problems, *e.g.*, their prevalence might be due to poor growth of coryneform bacteria. However, since the study of Maoz et al. (2003), these Gram-negative bacteria have been isolated from other traditional European cheeses, including farmhouse and industrially-produced cheeses (Mounier et al. 2005; Feurer et al. 2004a, 2004b; Rea et al. 2007; Ishikawa et al. 2007). Therefore, they may be considered as components of the cheese surface microbiota originating from the adventitious microbiota of the cheese-making environment. Their abundance in cheese and their role during cheese ripening have thus to be evaluated.

Fluorescence *in situ* hybridization (FISH) is a culture-independent approach for the analysis of complex microbial populations, for example in soil, sediments and activated sludge (Amann et al., 1995). It has also been used for biodiversity studies in food samples such as cheese (Ercolini et al. 2003a, 2003b; Cocolin et al. 2007), but these studies have mainly focused on the ecology of lactic acid bacteria. To our knowledge, this technique has not yet been applied for the characterisation of the yeasts and bacteria found at the surface of smear cheeses.

The aim of this study was to describe the microbial diversity of a red-smear cheese using culture-dependent and independent approaches. The yeast and bacterial diversity were studied using rRNA gene-based approaches. Probes readily available in the literature or designed in this study were then used to characterise *in situ* the yeasts and bacteria from the cheese surface using FISH analysis.

MATERIALS AND METHODS

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

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

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

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

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

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

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

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

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

FISH analysis

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

fluorescein or rhodamine.

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

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

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

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

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

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

RESULTS

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

Yeast diversity. Yeast cell count on the cheese surface was 2.4 x 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 ± 5% and 19.6 ±
261 6%, respectively. In contrast, culture-dependent analyses resulted in the identification of an

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

DISCUSSION

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

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

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

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

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

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

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

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

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

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

Acknowledgements

J. Mounier would like to thank INRA for awarding him a post-doctoral fellowship.

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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)	*Gamma-Proteobacteria*	GCCTTCCCACATCGTTT	23S (1027–1043)	35	Manz et al. (1992)
L-P-Grps-1901-a-A-18 (HGC69a)	*Actinobacteria*	TATAGTTACCACCGCCGT	23S (1901–1918)	25	Roller et al. (1994)
Yeast					
EUK516	Eukarya	ACCAGACTTGCCCTCC	18S (502–517)	20	Amann et al. (1990)
Ccat	*Candida catenulata*	TTTATCTCCCGCGCCT	26S (612-627)	20	This study
Cint	*Candida intermedia*	TTATCCACCCCTAGCA	26S (1415-1430)	20	This study
Geo	*Geotrichum* sp.	TTACGGGGCTGTCACCCT	26S (324-341)	20	This study
Ylip	*Yarrowia lipolytica*	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.
481

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	*Halomonas* sp. (AJ640133)	99
CC2[a]	FJ394919	*Microbacterium gubbeenense* (EU863414)	100
CC4[b]	FJ394925	*Vibrio litoralis* (DQ097524)	99
CC16[b]	FJ394920	*Hafnia alvei* (FM179942)	99
CC20[a]	FJ394921	*Leucobacter komagatae* (EU370411)	99
GC4[c]	FJ394922	*Lactococcus lactis* (AE006456)	99
GC27[c]	FJ394923	*Pseudoalteromonas* sp. (EU365474)	99
GC54[c]	FJ394924	*Arthrobacter arilaitensis* (AJ609626)	99

[a]Strains identified using the culture-dependent approach and cloning and sequencing of the 16S rRNA gene.
[b]Strains only identified in the culture-dependent approach
[c] Strains only identified using cloning and sequencing of the 16S rRNA gene.

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

501

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

502

503

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.

506

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

507 [a] 95% confidence interval

508

509

Figure legends.

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

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

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

Figure 1.

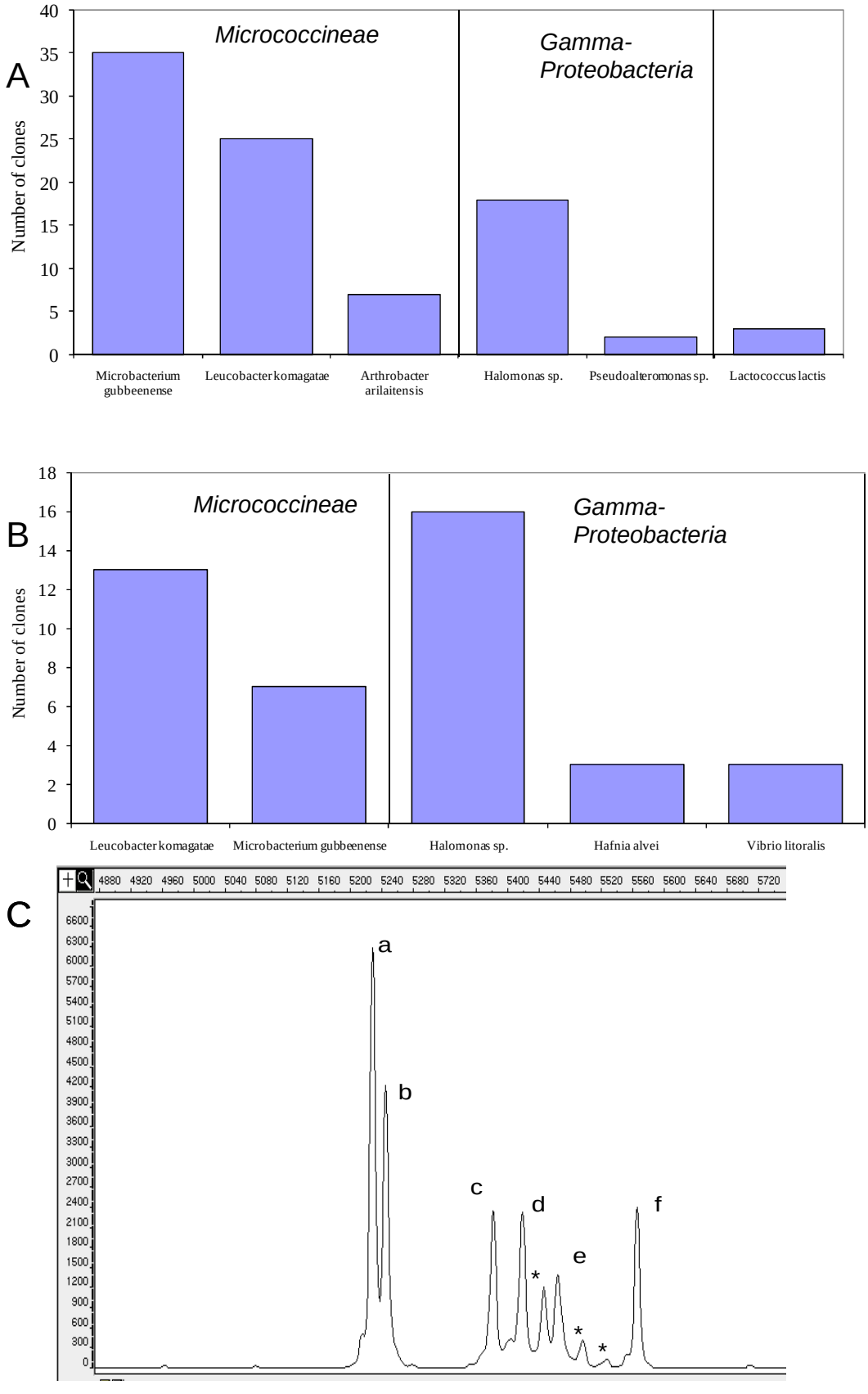
A
Micrococcineae
Gamma-Proteobacteria
Number of clones
0
5
10
15
20
25
30
35
40
Microbacterium gubbeenense
Leucobacter komagatae
Arthrobacter arilaitensis
Halomonas sp.
Pseudoalteromonas sp.
Lactococcus lactis
B
Micrococcineae
Gamma-Proteobacteria
Number of clones
0
2
4
6
8
10
12
14
16
18
Leucobacter komagatae
Microbacterium gubbeenense
Halomonas sp.
Hafnia alvei
Vibrio litoralis
C
a
b
c
d
e
f
*
*
*

Figure 2.

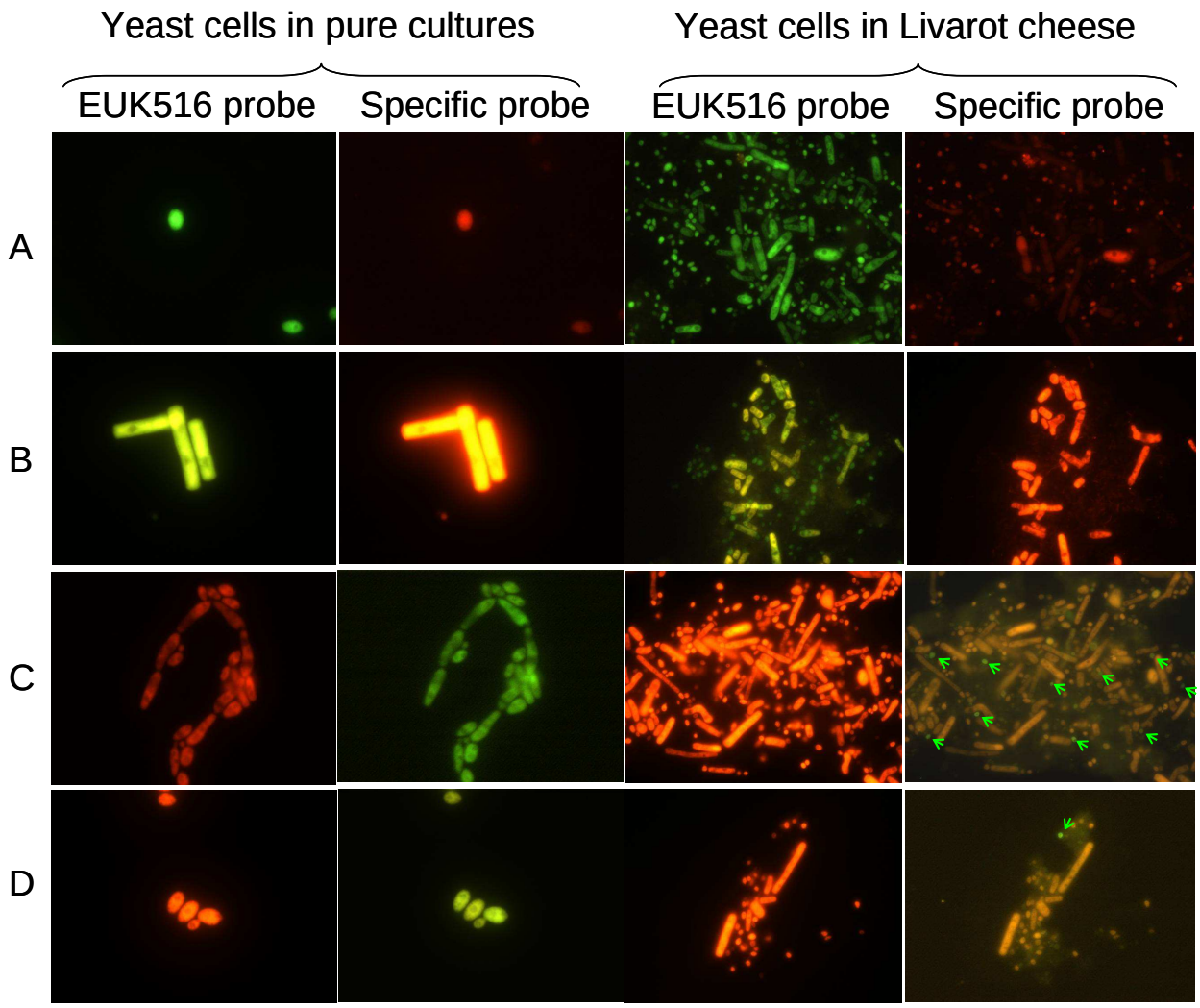
Yeast cells in pure cultures
Yeast cells in Livarot cheese
EUK516 probe
Specific probe
EUK516 probe
Specific probe
A
B
C
D

Figure 3.