

Culture-independent methods for identifying microbial communities in cheese

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1 **Culture-Independent Methods for Identifying Microbial**
2 **Communities in Cheese**

3
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1 **Abstract**

2

3 This review focuses on the culture-independent methods available for the description of both
4 bacterial and fungal communities in cheese. Important steps of the culture-independent
5 strategy, which relies on bulk DNA extraction from cheese and polymerase chain reaction
6 (PCR) amplification of selected sequences, are discussed. We critically evaluate the
7 identification techniques already used for monitoring microbial communities in cheese,
8 including PCR-ccdenaturing gradient gel electrophoresis (PCR-DGGE), PCR- temporal
9 temperature gradient gel electrophoresis (PCR-TTGE) or single-strand conformation
10 polymorphism-PCR (SSCP-PCR) as well as some other techniques that remain to be adapted
11 to the study of cheese communities. Further, our analysis draws attention to the lack of data
12 available on suitable DNA sequences for identifying fungal communities in cheese and
13 proposes some potential DNA targets.

14

1 **1. Microbial communities in cheese**

2

3 Cheese is produced throughout the world and more than 1000 varieties (Sandine and Elliker
4 1970) with different forms and flavours exist. Cheesemaking is conjectured to date back to
5 some 8000 years ago originating from the Middle East (Fox et al. 2000) where the first
6 fermented milk-based foods were made. Four basic ingredients are required to produce most
7 cheeses: milk, rennet, salt and microorganisms. These four ingredients are processed through
8 different steps such as acidification, coagulation, syneresis and ripening (see Fox et al. 2000).
9 Each unique combination of ingredients and processing parameters leads to a specific type of
10 cheese with unique properties. Fox et al. (2000) mentioned how fascinating it is that “such a
11 diverse range of products can be produced” from “basically similar raw material”. The
12 composition and activity of the microflora is the least controllable of all the parameters. The
13 microflora is made up of (i) starter lactic acid bacteria that are involved in acid production
14 during cheese manufacture and that also participate in the ripening processes to various
15 extents; and (ii) non-starter lactic acid bacteria, other bacteria, yeasts and filamentous fungi
16 that form the secondary microflora, which plays a significant role during ripening (Fox et al.
17 2000). Both starter and secondary flora modify the physical and chemical properties of
18 cheese, contributing to and reacting to environmental changes that occur during the
19 manufacture and ripening of cheese. Coppola et al. (2007) distinguished seven technological
20 production phases of cheese that may constitute selective pressures for the microbial species
21 that play an important role in community succession occurring throughout cheese
22 manufacture and ripening. The fundamental features that influence the dynamics of the
23 cheese ecosystem have been described by Beresford et al. (2001). They include (i) physical
24 features such as moisture, salt concentration, pH or redox potential which change during
25 cheese manufacture and are themselves influenced by the microflora, and (ii) biological

1 features such as those resulting from the interactions between microorganisms. The
2 characteristics of a given cheese therefore depend on microflora dynamics. Although food
3 microflora is undoubtedly not as diverse as environmental microflora, such as those found in
4 the soil (Garbeva et al. 2004), cheese microbial populations still remain difficult to control
5 due to their complex dynamics and to their interactions (Beresford et al. 2001). Knowledge of
6 the structure and dynamics of the whole microbial community of cheese would promote
7 better understanding of how cheese characteristics vary with respect to microbial growth and
8 metabolism. For instance, greater control over microflora composition would make it
9 possible to better select for specific organoleptic properties or to prevent quality defects or
10 spoilage. For these reasons, cheese bacterial and fungal communities have already been
11 partially identified using traditional methods and, to a lesser extent, molecular techniques.

12

13 **2. Towards culture-independent methods**

14

15 As in other fields in microbiology, species identification in cheese can be assessed through
16 the use of either culture-dependent or culture-independent methods. Culture-dependent
17 methods consist of isolating and culturing microorganisms prior to their identification
18 according to either morphological, biochemical or genetic characteristics. Different cultures
19 can even be bulked and analysed using global analysis methods, such as those as described
20 below for culture-independent methods (Ercolini et al. 2001; 2004). These methods have
21 already shed light on the structure of microbial populations during cheese manufacture
22 (Andrighetto et al. 1998; Fitzsimmons et al. 1999; Mannu et al. 2000; Berthier et al. 2001;
23 Dasen et al. 2003). However, culture-dependent methods are time-consuming, due to long
24 culture periods and elaborate culture techniques. They are therefore not amenable to
25 monitoring community dynamics during cheese manufacture and ripening. Moreover, species

1 occurring in low numbers are often out-competed *in vitro* by numerically more abundant
2 microbial species (Hugenholtz et al. 1998) and some species may be unable to grow *in vitro*
3 (Ward et al. 1990, 1992; Head et al. 1998). Hence, if culture conditions are poor and the
4 number of isolates too low, the culture collection will not be representative of the community
5 and the actual microbial diversity will be misinterpreted.

6 Community-level studies are relying more and more on culture-independent methods
7 based on the direct analysis of DNA (or RNA) without any culturing step. These methods are
8 based on protocols where total DNA (or RNA) is directly extracted from the substrate.
9 Coupled with a global analysis, these methods make it possible to study the total diversity
10 from the bulk extract in a single step. As they are fast and potentially more exhaustive, these
11 methods are well suited for analysing microbial communities over time and may provide the
12 possibility of exploring cheese microflora dynamics in detail. Most of these methods use
13 polymerase chain reaction (PCR) amplification of total DNA. The PCR amplicons from
14 different species are discriminated by using gel or capillary separation or by hybridization to
15 specific probes (**Figure 1**). However, these methods have potential biases, which will be
16 discussed below.

17

18 **3. 'Pick'em all'**

19

20 As previously mentioned, the isolation step of culture-dependent methods introduces biases
21 because some species are unable to grow under the selected experimental conditions. Culture-
22 independent methods typically aim at collecting DNA from the whole community to
23 overcome this bias. Nevertheless, technical issues may arise: DNA may not be recovered
24 from all genotypes or PCR amplification may be inaccurate. Some genotypes may remain
25 undetected due to low species abundance in the substrate, low species availability due to

1 insufficient homogenization of the matrix, inadequate cell lysis that prevents release of
2 nucleic acids, or inhibition of PCR amplification.

3 Complete homogenization of the cheese matrix can be challenging and partial
4 homogenization may hamper cell lysis, lowering DNA availability. Therefore, existing
5 protocols for DNA extraction from cheese include mechanical homogenization in salt
6 solutions such as trisodium-citrate (Coppola et al. 2006; Parayre et al. 2007). However, these
7 protocols may have to be adapted to the cheese under investigation. The extraction step is
8 usually performed in specific buffers, such as phosphate buffers containing various
9 detergents. Extraction can also be done with guanidine thiocyanate and *N*-laurylsarcosine
10 (Callon et al. 2006; Duthoit et al. 2005a,b; Delbès et al. 2007; Le Bourhis et al. 2007) and/or
11 lytic enzymes such as pronase (Florez and Mayo 2006; El Baradei et al. 2007) or proteinase
12 K (Ercolini et al. 2003; Cocolin et al. 2007). Lysis can be mechanically improved by
13 vigorously shaking samples in tubes containing beads (Randazzo et al. 2002; Duthoit et al.
14 2003, Duthoit et al. 2005a,b; Feurer et al. 2004a,b; Delbès and Montel 2005; Florez and
15 Mayo 2006; Delbès et al. 2007; Le Bourhis et al. 2007). New commercially available
16 automated instruments exploiting different techniques, such as pressure cycling technology,
17 which generates alternating hydrostatic pressure (Tao et al. 2007), or optimized mechanical
18 grinding, should be tested for their capacity to improve DNA extraction from microorganisms
19 in cheese.

20 Like for other food materials or environmental samples, DNA extraction yield and
21 PCR sensitivity are significantly reduced by a wide range of inhibiting substances (Wilson
22 1997). DNA extraction efficiency may be decreased by the high quantities of
23 macromolecules in cheeses, such as casein or lipids, that can adsorb detergents, chaotropic or
24 chelating agents necessary for nucleic acid extraction (Bonaïti et al. 2006). Moreover, even if
25 DNA yield is high, inhibitors that have not been eliminated may lower PCR sensitivity

1 (Wilson 1997). For example, Drake et al. (1996) reported that protein breakdown products
2 decreased the sensitivity of PCR amplification of rRNA genes of Lactobacillus spp.. DNA
3 solutions are therefore often purified using phenol-chloroform, which eliminates proteins and
4 various remnants (Randazzo et al. 2002, 2006; Cocolin et al. 2004; Feurer et al. 2004a,b;
5 Callon et al. 2006; Delbès and Montel 2005; Duthoit et al. 2003, 2005a,b; Delbès et al. 2007;
6 Le Bourhis et al. 2007). Several authors have used commercial DNA extraction kits based on
7 ion-exchange chromatography, size-exclusion or sorption techniques (Ercolini et al. 2001;
8 Rademaker et al. 2005) or just for DNA purification (Parayre et al. 2007).

9 It is noteworthy that most studies optimized DNA extraction to target bacterial DNA,
10 with the exception of Callon et al. (2006), who evaluated the dynamics of yeasts, and Florez
11 and Mayo (2006), who investigated the dynamics of the dominant microorganisms including
12 bacteria, yeasts and filamentous fungi. In order to characterise whole microbial communities,
13 protocols have to be adapted to extract DNA from all different types of microorganisms, as
14 attempted by Bonaïti et al. (2006).

15 Most culture-independent methods rely on PCR amplification of a targeted sequence.
16 This step can be affected by preferential or differential PCR amplification (Reysenbach et al.
17 1992; Walsh et al. 1992; Ercolini 2003; Kanawaga 2003) that may hinder the detection of
18 some genotypes when analysing bulk DNA extracted from cheese. Preferential PCR
19 amplification can be caused by (i) primer mismatches at the annealing sites of the DNA
20 templates of some genotypes or (ii) a lower rate of primer hybridization to certain templates
21 due to differential denaturation of these templates (Walsh et al. 1992; Suzuki and Giovannoni
22 1996). Systematically testing different sets of primers and enhancing DNA denaturation
23 during PCR by using different reagents (denaturants and cosolvents) may solve these
24 problems (Reysenbach et al. 1992; Weissensteiner and Lanchbury 1996; Hansen et al. 1998).
25 A second type of bias that may affect PCR carried out on complex bulk DNA extracts is the

1 occurrence of (i) heteroduplexes that arise in later PCR cycles when primer concentration
2 decreases and the concentration of PCR products is high enough to compete with the primers
3 for annealing (Kanawaga 2003) and (ii) chimeric amplicons that also form in later PCR
4 cycles when the concentration of incompletely extended primers is high enough to compete
5 with the original primer for annealing, or when template concentration is high enough to
6 allow the re-annealing of templates before or during primer extension (Kanawaga 2003). All
7 these artefacts can generate additional signals that do not correspond to genotypes in the
8 sample. These artefacts can be minimized by using a low number of PCR cycles.

9

10 **4. Analysing bulk DNA**

11

12 Through the construction of clone libraries from PCR products amplified from bulk cheese
13 DNA and subsequent sequencing of the different clones, it is theoretically possible to
14 examine the actual diversity of a given community. Although the cost of cloning and
15 sequencing is decreasing, this strategy remains expensive if used on a routine basis since it
16 implies large-scale cloning and sequencing to ensure that the community is exhaustively
17 screened. This strategy has been used for the study of bacterial cheese communities (Feurer et
18 al. 2004a,b; Delbès et al. 2007; El Baradei et al. 2007), but only as a complement to other
19 techniques (**Table 1**). Amongst the other molecular methods that may allow exhaustive
20 screening of microbial communities with no requirement for cloning PCR products, the most
21 commonly used in cheese microbiology are based on gel electrophoresis or chromatography.
22 The principal techniques that can be used to describe microbial communities in cheese are
23 briefly discussed below.

24

1 **4.1. PCR-denaturing gradient gel electrophoresis and PCR-temporal** 2 **gradient gel electrophoresis**

3
4 PCR-denaturing gradient gel electrophoresis (PCR-DGGE; Myers 1987) and PCR-temporal
5 temperature gradient gel electrophoresis (PCR-TTGE; Yoshino et al. 1991) (collectively
6 referred to here as PCR-DG/TTGE) are based on the separation of PCR amplicons of the
7 same size but with different sequences. In a denaturing acrylamide gel, DNA partially
8 denatures in discrete regions called melting domains. The melting temperature of these
9 domains is sequence specific. When the melting temperature (T_m) of the lowest melting
10 domain is reached, the DNA is partially denatured creating branched molecules. This
11 branching reduces DNA mobility in the gel. Therefore, amplicons of the same size but with
12 different nucleotide compositions can be separated based on differences in the melting
13 behaviour of their melting domains. For PCR-DGGE, the denaturing conditions rely on the
14 use of chemical denaturants (formamide and urea) incorporated into an acrylamide gel as a
15 linear denaturing gradient. PCR-DGGE electrophoresis is carried out at constant temperature,
16 typically between 55°C and 65°C (Ercolini 2004). For PCR-TTGE, the denaturing gradient is
17 obtained by varying the temperature over time without chemicals, thus generating more
18 reproducible data. PCR-DG/TTGE provides optimal resolution when PCR products are not
19 completely denatured. Thus, adding a so-called GC clamp (30-40bp) to one of the PCR
20 primers ensures that the PCR products are not completely denatured during the analysis
21 (Myers et al. 1985a,b; Sheffield et al. 1989). Prior to PCR-DG/TTGE analysis, the location of
22 the different melting domains within a DNA sequence can be predicted *in silico*, allowing
23 selection of the best primer pair. A PCR-DG/TTGE database containing migration profiles
24 corresponding to reference strains is generally created (Ogier et al. 2002, 2004) to facilitate
25 further identification of PCR-DG/TTGE profiles. A molecular ladder can be constructed by

1 using amplicons corresponding to representative species of this database and used in each gel
2 migration. However, this kind of database cannot be exhaustive and representative of the
3 actual community analysed and usually requires constant updates. The advantage of PCR-
4 DG/TTGE is that amplicons can be directly extracted from the DG/TTGE acrylamide gel and
5 sequenced. Unidentified profiles that do not match reference profiles can thus be sequenced,
6 compared to publicly available sequence databases and appended to the PCR-DG/TTGE
7 profile database. The first PCR-DGGE application to microbiology was done by Muyzer et
8 al. (1995) who studied bacterial communities of deep-sea hydrothermal vents. PCR-
9 DG/TTGE are the molecular techniques that have been the most extensively used for the
10 study of microbial communities in dairy products (e.g. Lafarge et al. 2004; Ogier et al. 2002;
11 2004) and more specifically to monitor the structure and even dynamics of microbial
12 communities in cheese (see references in **Table 1**).

13

14 **4.2. Single-strand conformation polymorphism-PCR**

15

16 Single-strand conformation polymorphism-PCR (SSCP-PCR; Orita et al. 1989; Ravnik-
17 Glavac et al. 1994) is a technique using either acrylamide gel- or capillary-based automated
18 sequencer, based on the separation of denatured (single-stranded) PCR products. Under non-
19 denaturing conditions, single-stranded DNA folds into tertiary structures according to their
20 nucleotide sequences and their physicochemical environment (e.g., temperature and ion
21 strength). This causes differences in electrophoretic mobility in non-denaturing gels. SSCP-
22 PCR is potentially easier to carry out than PCR-DG/TTGE since there is no need for gradient
23 gels or use of GC-clamp primers and it can be performed using an automated sequencer.
24 However, when using an automated sequencer, one of the disadvantages of this technique lies
25 in the difficulty of appending new data to an existing database: samples presenting unknown

1 profiles cannot be directly sequenced because they are labelled. SSCP-PCR is the second
2 most-used method to study microbial communities of cheese (see references in **Table 1**).

3

4 **4.3. Terminal restriction fragment length polymorphism**

5

6 Terminal restriction fragment length polymorphism (T-RFLP; Liu et al. 1997) is based on
7 digestion of fluorescent end-labelled PCR products with restriction endonucleases. Either one
8 or both 5' and 3' ends of the amplicon can be labelled by incorporating a dye on either one or
9 both PCR primers. The digested products are separated by electrophoresis using either
10 acrylamide gel- or capillary-based automated sequencer, with laser detection of the labelled
11 fragments. This system only detects the end-labelled terminal restriction fragments (TRFs) of
12 the digested PCR products and their size can be calculated based on the use of DNA size
13 standards that are run simultaneously with the samples. The data consists of the sizes of the
14 PCR amplicons that contain the labelled primer and are observed as electrophoregram peaks
15 or gel bands. Variation in the presence and location of the restriction sites results in different
16 genotypes having different TRF lengths. T-RFLP was initially developed as a fingerprinting
17 technique (Liu et al. 1997), with the number of TRFs used as an indication of biodiversity.
18 The use of a database of TRF profiles obtained from reference samples allows the
19 identification of the different species of a given community (Dickie et al. 2002). As for
20 SSCP-PCR performed using an automated sequencer, samples that present unknown profiles
21 cannot be directly sequenced because they are cut and labelled. T-RFLP has been used to
22 study diverse microbial communities (e.g. Liu et al. 1998) and has been extensively used by
23 mycologists since this method is reportedly more sensitive than PCR-DG/TGGE for fungi
24 (Brodie et al. 2003). However, some limitations—due in particular to inefficient restriction
25 enzyme cleavage—have been pointed out (Avis et al. 2006). T-RFLP was also found to be an

1 excellent tool for culture-independent assessment of bacterial community structure and
2 dynamics during ripening of cheese (Rademaker et al. 2005).

3

4 **4.4. Denaturing high-performance liquid chromatography**

5

6 Denaturing high-performance liquid chromatography (DHPLC) (Oefner and Underhill 1995
7 in Xiao and Oefner 2001) allows separation of amplicons using an ion-pair reversed-phase
8 high-performance liquid chromatography (IP RP HPLC) automated detection system. It was
9 initially used to detect single nucleotide polymorphisms (SNPs) in clinical applications (e.g.,
10 Frueh and Noyer-Weidner 2005) based on the analysis of heteroduplex formation. DHPLC is
11 a promising approach for microbial community analysis (Barlaan et al. 2005). PCR
12 amplicons are injected into a chromatography column containing alkylated non-porous
13 polystyrene/polydivinylbenzene particles. Separation of the different amplicons relies on the
14 elution of partially denatured PCR products, which is achieved with the intervention of the
15 ion-pairing agent, triethylammonium acetate (TEAA) and the cartridge matrix of the system.
16 As described in Barlaan et al. (2005), under partial denaturation and a given flow rate of
17 gradient buffers, amplicons of the same size but with different melting behaviours due to
18 different nucleotide compositions will have different retention times. These differences are
19 due to the reduced negative charges in the single-stranded portions of the partially denatured
20 PCR products compared to the double-stranded molecules. The negative charges interact with
21 the positive charges of ammonium ions of TEAA, and double-stranded DNA is more
22 efficiently adsorbed to the stationary phase in the cartridge. DHPLC permits high-throughput
23 automated analyses and, unlike SSCP-PCR or T-RFLP, it allows the collection of elution
24 fractions corresponding to different amplicons that can be directly sequenced even more
25 easily than with PCR-DG/TTGE methods. Protocols have already been developed for

1 analysing marine bacterial populations (Barlaan et al. 2005), monitoring intestinal microflora
2 (Goldenberg et al. 2007) and, recently, also for studying the bacterial diversity occurring in
3 natural whey cultures used for cheese manufacture (Ercolini et al., 2008). The latter study
4 showed that DHPLC technique was at least as effective as the widely used PCR-DGGE
5 technique in assessing species diversity of food-related microbial communities.

6

7 **4.5. DNA microarrays**

8

9 The complementary DNA (cDNA) microarray (or microchips) technology has dramatically
10 changed the way gene expression can be assessed (Duggan et al. 1999). Since Guschin et al.
11 (1997) introduced the DNA microarray approach to microbial community analysis, the DNA
12 array-based methods hold great promise for more extensive analyses of microbial
13 communities (Zhou et al. 2003; Bodrossy et al. 2007; Wagner et al. 2007) and potential
14 applications cover various fields of microbiology, including food science (Bae et al. 2005).
15 By using DNA microarrays, the identification of labelled PCR products or directly retrieved
16 RNA relies on their hybridization to oligonucleotide probes attached to a substrate. A
17 description of the various types of microarrays is given by Zhou et al. (2003). Unlike the
18 previously described methods, DNA array technology potentially allows the simultaneous
19 application of a nearly unlimited number of probes in a single hybridization experiment
20 (Small et al. 2001; Peplies et al. 2003). This technique is therefore very well suited to even
21 the most complex environmental samples. However, for this approach to work, each probe
22 must specifically hybridize, under given stringency conditions, to a fully matched DNA
23 target (Valinsky et al. 2002) which has been proven very difficult (Wagner et al. 2007).
24 Moreover, the design and refinement of efficient probes depend on the comprehensiveness
25 and quality of probe target database (Wagner et al. 2007). The low quality of some annotated

1 sequences in the available databases complicates probe design. Nonetheless, given the lower
2 diversity of food-borne communities (compared to environmental communities) and given
3 the unequalled high-throughput capacity of DNA microarrays, it would be worthwhile to
4 devote research efforts for developing a robust probe target sequence database necessary for
5 using DNA microarrays as a routine tool for monitoring cheese microbial communities
6 throughout cheese manufacture.

7

8 **5. What is a good target?**

9

10 The discrimination between the different species from a cheese sample depends on the
11 capacity of the analytical technique to reveal polymorphisms. However, whatever analytical
12 technique is used, no discrimination is possible without polymorphism. The target DNA
13 sequence must therefore be variable between species. In addition, since almost all of the
14 above-described techniques rely on PCR amplification, the target DNA sequence must also
15 include conserved sequences that can serve as anchors for PCR primers.

16 Unlike prokaryotic genes that do not usually harbour introns, fungal genes are
17 interrupted by non-coding introns. Although introns are much more variable than exons and
18 thus offer high levels of polymorphism between species, their length and their location vary
19 greatly beyond the family level (James et al. 2006). It is therefore difficult to design primers
20 that allow PCR amplification of introns from all the species in the community. Moreover, the
21 analysis of exons is often discriminative enough to distinguish between species since they
22 show sufficient variation at the interspecific level, especially at their third codon positions
23 that evolve at rates similar to introns. Consequently, most of the DNA regions selected for
24 studying fungal communities are located in exons.

25

1 **5.1 Bacterial and fungal ribosomal DNA**

2

3 Since Woese and Fox's paper (1977), most community surveys have focused on RNA genes
4 and intergenic spacers. Bacterial 16S, 23S and 5S rRNA genes are organized into a co-
5 transcribed operon. From one to as many as 15 copies of the operon may be dispersed in a
6 bacterial genome (Klappenbach et al. 2000). In contrast, in fungi and more generally in
7 eukaryotes, 18S, 28S 5.8S and 5S rRNA genes and intergenic spacers are tandemly repeated
8 (up to hundreds of repeats) (Cihlar and Sypherd 1980). The 5S rRNA is, in some species,
9 separated from the main unit (Kellog and Appels 1995). These repeated units are organized
10 as multiple and dispersed arrays at chromosomal nucleolar organizer regions (NORs). In both
11 prokaryotes and eukaryotes, rRNA genes usually show extraordinary sequence homogeneity
12 within a species (Elder and Turner 1995; Liao 1999). This sequence homogenization is likely
13 to be due to a process known as concerted evolution. The underlying mechanisms of
14 concerted evolution involve recombination processes collectively referred to as mitotic or
15 somatic recombination (see Liao 2000) including unequal crossing-over and gene conversion
16 that are responsible for DNA repair during replication and transcription. The paradigm of
17 concerted evolution makes it possible to treat repeated rRNA genes as one locus. For this
18 reason, rRNA genes have been widely used in phylogeny reconstructions and species
19 identification. However, deviations from this expected homogeneity of rDNA sequences have
20 been detected in many taxa.

21 Although multiple-gene investigation has already been recommended for bacterial
22 species identification (Stackebrandt et al. 2002), studies evaluating bacterial diversity and/or
23 dynamics or investigating bacterial phylogenetics have mostly focused only on ribosomal
24 DNA array analysis (Throbäck et al. 2004). In cheese, all bacterial community surveys are
25 based on the analysis of 16S rRNA genes and 16S-23S intergenic region (**Table 1**). Bacterial

1 16S rRNA genes comprise nine hypervariable regions, V1-V9, that exhibit considerable
2 sequence diversity among species (Van de Peer et al. 1996; Baker et al. 2003). These
3 hypervariable regions are generally flanked by conserved sequences that can serve as anchors
4 for universal or specific primer pairs (see Baker et al. 2003). They are therefore used for
5 species identification and allow the evaluation of community diversity. No single region can
6 differentiate among all bacteria and different regions can be used depending on the goal
7 (Chakravorty et al. 2007). In cheese, the V3 hypervariable region is the target that has been
8 the most extensively used, but the use of different sets of primers targeting different regions
9 can improve the analysis (Delbès et al. 2007).

10 In fungi, the analysis of fungal rRNA genes limits identification to the genus or
11 family level (Anderson and Cairney 2004). The fungal internal transcribed spacers (ITS)
12 provide a greater taxonomic resolution than rRNA genes and are generally used for fungal
13 community surveys in different environments (Anderson and Cairney 2004). The ITS is a
14 region located between the 18S rRNA and 28S rRNA genes and including the 5.8S rRNA
15 gene that splits the ITS into two parts: ITS1 and ITS2. The ITS region undergoes a faster rate
16 of evolution than rRNA genes but its sequence remains homogenous within a species. Indeed,
17 both ITS1 and ITS2 fulfil significant functions during rRNA maturation (Joseph et al. 1991;
18 Liu and Schardl 1994) and are under selective pressure. In Penicillium spp. which are
19 amongst the most prominent fungi in cheese, the analysis of ITS affords better discrimination
20 than rRNA genes (Skouboe et al. 2000; Doaré-Lebrun et al. 2006). Nevertheless, Doaré-
21 Lebrun et al. (2006) when analysing the fungal community in grape showed that the use of
22 ITS was not sufficient for good interspecific discrimination, especially for the species of the
23 subgenus Penicillium, a monophyletic group of moulds that represent 58 of the ≈250
24 accepted species in the genus *Penicillium* (Seifert et al., 2007). It is therefore recommended

1 to use other targets in addition to ITS when analysing fungal communities in cheese where
2 species of this subgenus are among the most prominent.

3

4 **5.2. Other fungal genes**

5

6 With the exception of rRNA genes and ITS, only a few number of sequences have been used
7 for studying fungal communities. Recently, Seifert et al. (2007) tested the reliability of using
8 the *COI* gene that codes for mitochondrial cytochrome *c* oxidase 1 as a barcoding tool for
9 Penicillium spp. identification. This gene has already been used extensively in animal
10 barcoding and could be used as a new marker to investigate fungal communities. Sequence
11 analysis of *COI* yielded a coherent phylogeny of the taxonomically challenging Penicillium
12 subgenus (Seifert et al. 2007), suggesting that *COI* is a powerful tool for fungal barcoding.
13 However, the results by these authors show that *COI* is not more variable among the species
14 of the subgenus Penicillium than ITS. The low *COI* interspecific divergence precludes
15 distinguishing species of the subgenus Penicillium using gel/capillary separation of PCR
16 products.

17 Amongst other possible targets, genes that encode mitochondrial rRNA genes
18 (Lutzoni et al. 2004) could represent an alternative to nuclear rRNA genes. Moreover, the
19 general effort for inferring phylogeny of the kingdom fungi (Lutzoni et al. 2004; Blackwell et
20 al. 2006; Hibbet et al. 2007) has yielded a high number of sequences for different genes and
21 for different fungal species that can be tested for species identification. The non-ribosomal
22 genes used for inferring fungal phylogenies comprise: (i) the genes *RPB1* and *RPB2* that
23 encode the two largest subunits of the RNA polymerase II (James et al. 2006); the gene *EF-1*
24 α coding for elongation factor 1-alpha (James et al. 2006); the gene *BenA* encoding β -tubulin
25 A (Einax and Voigt 2003; Samson et al. 2004); the *GPD* gene coding for glyceraldehyde 3-

1 phosphate dehydrogenase (Berbee et al. 1999). All these potential targets should be tested for
2 their usefulness for distinguishing between different species of fungi that compose fungal
3 communities in cheese. According to Samson et al (2004) and Seifert et al (2007) *BenA* is
4 more variable than ITS amongst the species of the Penicillium subgenus and could be a good
5 target for analysing cheese fungal communities using methods based on gel/capillary analysis
6 of PCR products. The most promising sequences to analyse can be identified by *in silico* data
7 mining of publicly available databases. However, sequence availability for a large range of
8 species varies amongst genes. Additional sequencing is likely to be required to cover a
9 reasonable array of species. This requires selecting and designing universal or even a set of
10 different primers to sequence the targeted genes in the different species investigated.

11

12 **6. Limitations and pitfalls of culture-independent methods**

13

14 As previously mentioned, the first limitation arises from the difficulty of accessing every
15 genotype from the community: poor DNA extraction yield, PCR inhibition by various
16 extraction by-products or by substances coming from the cheese matrix itself, and differential
17 PCR amplification have already been reviewed (Wintzingerode et al. 1997; Ercolini 2004).
18 We have already made some suggestions to address, when possible, these concerns. The
19 various techniques have limitations in terms of resolution: PCR-DG/TTGE, SSCP-PCR, T-
20 RFLP, DHPLC and even microarrays can generate patterns in which different genotypes
21 group together due to co-migration/co-elution (Ogier et al. 2002; Feurer et al. 2004a; Lafarge
22 et al. 2004; Delbès et al. 2007; El-Baradei et al. 2007) or ‘co-hybridization’ (Wagner et al.
23 2007). Since they allow direct (cloning) sequencing of migrants/eluants, PCR-DG/TTGE and
24 DHPLC may overcome the co-elution/co-migration problem more easily. Another limitation
25 of gel/capillary migration-based methods is obtaining profiles in which the less-common

1 amplified sequences cannot be distinguished from background noise (Feurer et al. 2004a;
2 Callon et al. 2006). This problem increases with the diversity of the community. Ideally,
3 multiple DNA targets should be used, as suggested by Doaré-Lebrun et al. (2006) and Delbès
4 et al. (2007), to increase the efficiency in discriminating between species. Another remark
5 made by several authors (Ercolini et al 2001; Feuerer et al. 2004a,b; Florez and Mayo 2006;
6 Delbès et al. 2007) is that culture-independent methods regularly fail to identify species
7 obtained using culture-dependent methods. These two types of methods reveal different
8 images of the same community. Therefore, those authors suggest that using a polyphasic
9 approach, combining culture-dependent and culture-independent methods, may be
10 worthwhile to obtain a more accurate view of the structure of the microbial community.
11 Nevertheless, culture-independent methods have proven to be the only ones with the capacity
12 for monitoring the rapid dynamics of microbial communities during cheese manufacture and
13 ripening processes, where microorganisms encounter multiple environmental shifts. Culture-
14 independent methods need to be improved to reveal as accurately as possible the actual
15 microbial communities.

16

17 **7. Microbial community activity in cheese**

18 In order to reveal metabolically active populations, some authors performed analysis on
19 reverse-transcribed RNA. Randazzo et al. (2002) and Duthoit et al. (2005a) were among the
20 first to use reverse transcriptase-PCR-DGGE (RT-DGGE) and reverse transcriptase-PCR-
21 SSCP (RT-PCR-SSCP), respectively. By combining RT-PCR-DGGE and PCR-DGGE or
22 RT-PCR-SSCP and PCR-SSCP, these authors were able to differentiate the active component
23 (rRNA-derived) from the total diversity (rDNA-derived) of the community. Duthoit et al.
24 (2005b) even attempted to link population dynamics and activities (as assessed by PCR-
25 SSCP and RT-PCR-SSCP) to sensorial characteristics in order to identify which species play

1 a major role in the development of the organoleptic properties of Salers cheese. However,
2 their analysis failed to explain the diversity of sensorial qualities in Salers cheese.
3 Nonetheless, combining DNA- and RNA-based analyses with more quantitative methods,
4 such as DNA- and cDNA-microarrays coupled with chemiometric and/or sensorial tests,
5 could significantly increase our ability to identify the impact of the microbial population on
6 organoleptic characteristics.

7

8 **8. Perspectives in the field of cheese microbiology**

9 Our review draws attention to the fact that only a few studies (Florez and Mayo 2006; Callon
10 et al. 2006; Gente et al. 2007) have targeted fungal communities of cheeses. However, yeasts
11 and filamentous fungi play a vital role in the development of organoleptic characteristics
12 and/or, on the contrary, may be the source of quality defects. Protocols need to be developed
13 to optimize fungal DNA extraction from cheese and new target sequences, in addition to
14 ribosomal loci, should be investigated to better discriminate between the different fungal
15 species found in cheese. Surveys could then be extended to the whole microbial community.

16 It is noteworthy that, at present, some species can only be detected by culture and that
17 polyphasic methods, including both culture-independent and culture-dependent approaches,
18 are necessary to at least allow evaluation of the efficiency of these two types of approaches.
19 Nevertheless, although culture-independent methods still fail to exhaustively describe the
20 microbial community composition in cheese, these methods provide a much faster
21 assessment of community composition than culture-dependent methods do. New light has
22 been shed on population dynamics and culture-independent methods offer a powerful tool for
23 controlling cheese manufacture. Therefore, it is worthwhile to devote efforts to improve the
24 resolution of culture-independent methods and facilitate their transfer to cheese industry.

1 Because cheese microbial communities are much less complex than environmental
2 communities (in terms of the number of species encountered), they offer an ideal system to
3 test identification methods that require setting up reference databases or designing individual
4 nucleic probes, such as for DNA microarrays. A major task is to select the best targets
5 allowing both universal screening of the microorganisms in cheese and discrimination
6 between taxa at the species level. Achieving this goal requires increasing the accessibility of
7 high-throughput DNA sequencing technology that can provide digital images of cheese
8 ecosystems (cheese ecogenome) by identifying genes associated with species and even
9 functions.

10

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12

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17

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