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Abstract

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

1. Microbial communities in cheese

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

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

2. Towards culture-independent methods

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

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

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

3. 'Pick'em all'

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

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

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

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

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

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

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

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

4. Analysing bulk DNA

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

4.1. PCR-denaturing gradient gel electrophoresis and PCR-temporal temperature gradient gel electrophoresis

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

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

4.2. Single-strand conformation polymorphism-PCR

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

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

4.3. Terminal restriction fragment length polymorphism

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

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

4.5. DNA microarrays

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

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

5. What is a good target?

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

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

5.1 Bacterial and fungal ribosomal DNA

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

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

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

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

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

5.2. Other fungal genes

With the exception of rRNA genes and ITS, only a few number of sequences have been used for studying fungal communities. Recently, Seifert et al. (2007) tested the reliability of using the *CO1* gene that codes for mitochondrial cytochrome *c* oxidase 1 as a barcoding tool for Penicillium spp. identification. This gene has already been used extensively in animal barcoding and could be used as a new marker to investigate fungal communities. Sequence analysis of *CO1* yielded a coherent phylogeny of the taxonomically challenging Penicillium subgenus (Seifert et al. 2007), suggesting that *CO1* is a powerful tool for fungal barcoding. However, the results by these authors show that *CO1* is not more variable among the species of the subgenus Penicillium than ITS. The low *CO1* interspecific divergence precludes distinguishing species of the subgenus Penicillium using gel/capillary separation of PCR products.

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

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

6. Limitations and pitfalls of culture-independent methods

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

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

7. Microbial community activity in cheese

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

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

8. Perspectives in the field of cheese microbiology

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

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

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

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