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A review of the molecular approaches to investigate the diversity and activity of cheese microbiota

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Abstract The cheese microbiota is characterized by the presence of a large variety of bacteria, yeasts, and molds, and many factors influence their growth and survival. The microbial community in cheese at various stages of ripening has been extensively studied by microbiological techniques based on the cultivation of the microorganisms on media and phenotypic or genotypic characterization of a fraction of the community (culture-dependent methods). Culture-independent methods based on DNA or RNA extraction offer the possibility of profiling uncultivable members of the microbial community as well as distinguishing those that are metabolically active. In this review, the status of research on available molecular tools used to characterize the microbiota in the cheese matrix are described and discussed in order to assess the metabolic functionality of the microbial community, its diversity, as well as the identification of species and their comparative quantification. Combining culture-dependent and culture-independent approaches can contribute to improving the strain selection process by understanding the basis of technological performance. Defined starter and adjunct cultures will improve and standardize cheese quality and safety. Future perspectives include the application of methods such as high-throughput quantitative reverse transcription PCR and pyro-sequencing to quantify the contribution of the microbial community to cheese ripening.

分子方法研究干酪微生物群体多样性和活力——综述

摘要 干酪微生物区域中存在多种细菌、酵母和霉菌，多种因素影响着上述微生物的生长和存活。采用基于微生物在培养基中培养以及群体中部分微生物的表型或遗传型特征的微生物研究技术，已经对干酪成熟的不同阶段中微生物群体，进行了深入研究。基于DNA或RNA提取的非培养方法提供了研究微生物群体中非培养物以及区分那些代谢活跃的微生物的可能。本文中描述和讨论了当前可用于研究干酪中微生物区系的分子工具的研究现状，旨在评价微生物群体的代谢功能性、多样性以及种属的鉴定、相应的定量方法。基于技术特性的理解，培养和非培养研究方法的结合能够改善菌株的选择过程。明确发酵剂和附属发酵剂将改善和标准化干酪的质量和安全性。同时对应用高通量的定量反转录PCR、焦磷酸测序等方法量化微生物群体对干酪成熟的贡献进行了展望。

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关键词 干酪 · 干酪成熟 · 微生物生态系统 · DNA特性 · rRNA探针 · 非培养方法 · 未培养微生物 · 代谢活跃微生物

Abbreviation

ARDRA	Amplified ribosomal DNA restriction analysis
ARISA	Automated rDNA internal spacer analysis
DGGE	Denaturing gradient gel electrophoresis
FISH	Fluorescence in situ hybridization
FRAP-PCR	Fluorescent RNA arbitrarily primed PCR
LH-PCR	Length heterogeneity polymerase chain reaction
MLSA	Multilocus sequence analysis
MLST	Multilocus sequence typing
PFGE	Pulsed-field gel electrophoresis
qPCR	Quantitative PCR
RAPD	Randomly amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism (RFLP)
RT-PCR	Reverse transcription PCR
RT-qPCR	Reverse transcription quantitative PCR
SSCP	Single-strand conformation polymorphism
SSH	Suppression subtractive hybridization
TGGE	Temperature gradient gel electrophoresis
T-RFLP	Terminal restriction fragment length polymorphism
VBNC	Viable but non-cultivable

1 Introduction

Cheese is characterized by the presence and succession of numerous microorganisms such as bacteria, yeasts, and molds which interact to play a major role during manufacturing and ripening. Cheese is the oldest preservation form of milk even if it is now more appreciated for its taste and flavor. Fox et al. (2000) indicated four basic ingredients required to produce most cheeses: milk, rennet, salt, and micro-organisms. These four ingredients are processed through different steps of acidification, coagulation, syneresis, and ripening (Fox and Cogan 2004; Fox et al. 2000). The categories and/or the classification of cheeses are usually specified without taking into account the microbial diversity or activities in situ of the cheese matrix (Coppola et al. 2008). For instance, according to their texture, cheeses are classified as “hard, semi-hard, or soft.” According to the ripening time, in some cases, cheeses are called “fresh, unripened, or ripened” (Coppola et al. 2008; Mucchetti and Neviani 2006). Cheese is manufactured either with the combination or with the addition of starter cultures (Giraffa and Neviani 2001). Mucchetti and Neviani (2006) enumerated some categories of cheeses as follows: (1) cheeses produced with pasteurized milk and selected starter; (2) cheeses produced with pasteurized milk and natural starter; (3) cheeses produced with thermal-treated milk

and natural starter; (4) cheeses produced with raw milk and selected starter; (5) cheeses produced with raw milk and natural starter; (6) cheeses produced only with raw milk. On a similar basis, McSweeney et al. (2004) reported different classification schemes of cheeses. None of the described classifications or categories of cheeses have considered the microbial diversity characterizing different types of cheeses. Today, it is notoriously shown that cheese is a microbiologically dynamic food, hosting diverse metabolically active bacteria, yeasts, and molds.

The cheese microbiota is dependent on factors that influence microbial growth and survival (microbiota of raw milk, acidification process, and storage conditions; Beresford et al. 2001; Beresford and Williams 2004; Irlinger and Mounier 2009). Fox et al. 2000 described three types of microbiota: primary starter, secondary cultures, and non-starter bacteria. Cheese starter cultures are involved in acid production during cheese manufacture and also participate in the ripening process to various extents in combination with ripening cultures. Non-starter lactic acid bacteria (NSLAB), other bacteria, yeasts, and filamentous fungi originating from milk or the processing environment also play a significant role during ripening. Coppola et al. (2008) have compiled and excellently summarized the most important species of the microbiota occurring during the manufacture of the main types of cheese. Previous reviews on starter lactic acid bacteria (SLAB) and NSLAB comprise those of Leroy and De Vuyst (2004), Beresford and Williams (2004), and McSweeney et al. (2004).

The presence of starter and ripening cultures in cheese microbiota is not sufficient to explain flavor formation in raw milk cheese. For example, artisanal cheeses are characterized by an indigenous microbial ecosystem responsible for their quality and playing an important role in developing cheese flavor, appearance, and texture (Randazzo et al. 2002). Otherwise, flavor formation and improvement is determined by complex dynamics and interactions between microorganisms which are responsible for variations in cheese quality. So, for these reasons, if no starter cultures are used, it is necessary to investigate the composition of microbial communities during the production process of artisanal cheese because they remain difficult to control (Fuka et al. 2010). This specific aspect related to the study of lactic acid bacteria (LAB) in artisanal cheese has been covered in depth by Randazzo et al. (2009). Finally, the microbiota of any type cheese is influenced by the source and treatment of the cheese milk, the manufacturing process, and the hygienic practices observed during milking, cheese making, and ripening (Arteau et al. 2010; Beresford et al. 2001; Martin-Platero et al. 2008).

The taxonomic structure of the microbial community in cheese at various stages of ripening has been studied by microbiological techniques based on the cultivation of the microorganisms on media and phenotypic or genotypic characterization of a fraction of the community (culture-dependent methods; Beresford et al. 2001; Coeuret et al. 2003; Ward and Roy 2005). Culture-independent techniques based on direct analysis of DNA or RNA have been developed for characterization of the microbial community and evaluation of in situ gene expression and determination of metabolic activities of the cheese microbiota (Bonaïti et al. 2006; Giraffa and Carminati 2008; Giraffa and Neviani 2001; Jany and Barbier 2008; Monnet et al. 2008; Randazzo et al. 2009; Ulve et al. 2008). Jany and Barbier (2008) amply reviewed the utility of culture-independent methods for identifying microbial communities in cheese, where the compilation of most papers focused on using

bacterial and fungal ribosomal DNA as a good target. These authors also introduced the importance of revealing metabolically active populations by the analysis of reverse-transcribed RNA. Furthermore, according to these authors, the combination of DNA- and RNA-based analysis with more quantitative methods via a polyphasic approach could significantly increase our ability to identify the impact of the microbial population on organoleptic characteristics. Coppola et al. (2008) concluded their noteworthy review by recommending the use of quantitative PCR systems coupled with the appropriate procedure of RNA extraction from the cheese matrix to better understand which of the important activities are being carried out in certain steps of cheese production.

During the most important steps of cheese making, molecular approaches offer the possibility to identify and quantify both the microbial species encountered in each phase and their activity as well as strain diversity. By targeting the rRNA genes and evaluating the expression of functional genes as related by recent papers (Florez and Mayo 2006a; Nocker et al. 2007), genomic sequencing and transcriptome analysis could support discrimination and quantification of viable and active microorganisms of the cheese matrix in situ. Proteomics and metabolomics are complementary approaches providing endpoint analyses of complex microbial processes. The final judge of cheese acceptability will still be sensory analysis, but combining these approaches will lead to predictive markers and a better understanding of the performance parameters for each phase of cheese making.

Therefore, this review aims to provide an overview of the molecular techniques useful to describe and analyze the microbial community with a focus on RNA-based methods targeting microbial activity. In this respect, the assessment of the metabolic functionality, the diversity of the cheese microbial community, as well as the in situ gene expression of the cheese matrix microbiota will be highlighted. The identification of species and their comparative quantification will also be covered. This information will be useful for improving cheese flavor and quality by understanding metabolic behavior and changes in the microbial community during cheese ripening.

2 Culture-dependent and culture-independent methods

The development of molecular methods has transformed cheese microbial ecology. Their application to the cheese matrix helps give significant insight into specific isolates, microbial populations, and into the evolution and the nature of the microbial groups during cheese ripening (Bonaïti et al. 2006). The dynamics and the structure of the whole microbial community of cheese are a step toward promoting better understanding of how cheese characteristics vary with respect to microbial growth and metabolism. Therefore, some studies are focused on developing traditional cultivation methods to partially identify and control cheese microbiota while others target the molecules intrinsic to the microorganisms, bypassing traditional cultivation. Those are culture-dependent (a in Fig. 1) and culture-independent methods (b and c in Fig. 1), respectively. The combination of both approaches can be used to describe the contribution of individual microorganisms found in cheese (culture-dependent) and to determine the diversity and dominance of microbial

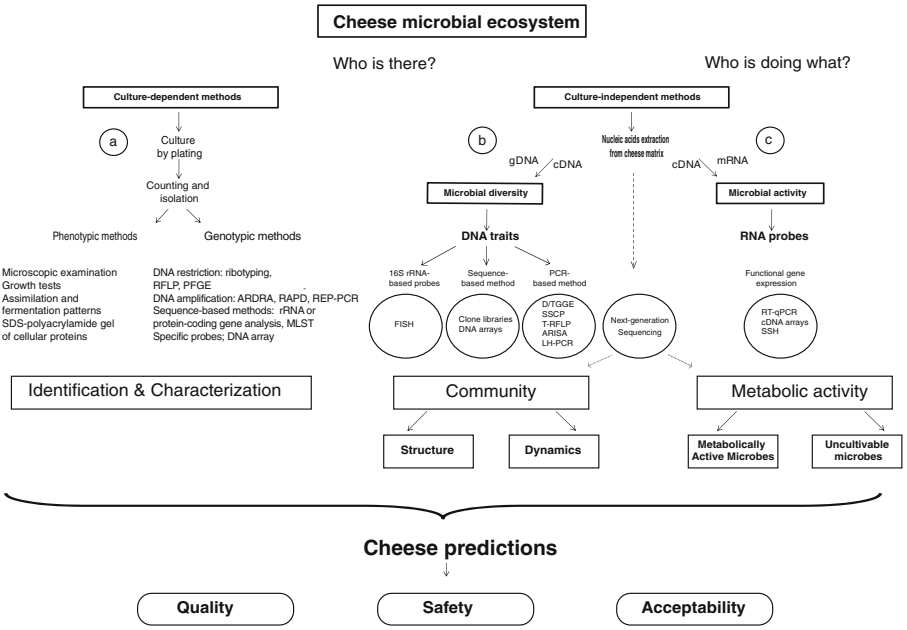

Fig. 1 Flow diagram of the culture-dependent and culture-independent methods to study the community structure and activity of cheese microbiota. *a* Culture-dependent methods. *b*, *c* Culture-independent methods for microbial diversity (*b*) and microbial activity (*c*). *RFLP* restriction fragment length polymorphism, ribotyping, *PFGE* pulsed-field gel electrophoresis, *ARDRA* amplified ribosomal DNA restriction analysis, *RAPD* randomly amplified polymorphic DNA, *REP-PCR* repetitive extragenic palindromic PCR, *ARISA* automated rDNA internal spacer analysis, *DGGE* denaturing gradient gel electrophoresis, *FISH* fluorescence in situ hybridization, *ISH* in situ hybridization, *LH-PCR* length heterogeneity polymerase chain reaction, *qPCR* quantitative PCR, *RT-PCR* reverse transcription PCR, *RT-qPCR* reverse transcription quantitative PCR, *SSCP* single-strand conformation polymorphism, *SSH* suppression subtractive hybridization, *TGGE* temperature gradient gel electrophoresis, *T-RFLP* terminal restriction fragment length polymorphism

populations or species (culture-independent) as well as microbial activity (Delbes et al. 2007; Ercolini et al. 2001; Mounier et al. 2009; Randazzo et al. 2010).

2.1 Culture-dependent methods

Ercolini et al. (2001) defined the culture-dependent methods as those which consist of isolating and culturing strains prior to their identification according to either phenotypic or genotypic characteristics (a in Fig. 1; Jany and Barbier 2008). In natural environments such as cheese, these methods are not equally applicable to all types of cheese. Hence, efforts should be made to establish the most suitable method or combination of methods to be applied to any type of cheese (Ercolini et al. 2001; Martin-Platero et al. 2009). Only a small fraction of microorganisms can be retrieved on culture media by semi-randomized modes of colony picking, and often, the isolates do not seem to represent the real taxonomic inventory of microorganisms with active gene expression in cheese (Coppola et al. 2001; Martin-Platero et al. 2009; Van Hoorde et al. 2010a). In addition, culture-based techniques are time-consuming due to long culture periods, and some species are either unable to grow in vitro or

often outcompeted by numerically more abundant microbial species (Muyzer et al. 1993). Hence, if culture conditions are restrictive or competitive and the number of isolates too low, the culture collection will not be representative of the cheese microbiota and the actual microbial diversity will be underestimated. They are therefore not completely sufficient for monitoring microbial dynamics during cheese manufacture and ripening. Hence, culture-dependent methods are limited not only by the available media used for the characterization of naturally occurring bacterial species or the study of the equilibrium of the biota present during the ripening process (Denis et al. 2001) but also by the lack of investigation in this field. The number of SLAB could be underestimated during ripening due to the intrinsic limits of the agar plate count technique. A study performed to investigate cultivable and total lactic microbiota in Parmigiano Reggiano cheese showed that the highest LAB counts were recovered on media simply prepared from whey or ripened cheese to reproduce the natural composition, facilitating the growth of strains arising from natural whey starter. The nutritionally demanding microorganisms found the best conditions for growth in these media (De Dea Lindner et al. 2008). For strains of NSLAB which have high nutritional demands, rich media with milk sugar such as whey agar medium (WAM) was not as reliable as cheese agar medium (CAM) made from ripened cheese. The chemical composition of CAM showed a high amount of digested proteins and NaCl and the absence of milk sugars. This medium rich in peptides and amino acids is well adapted to microorganisms demonstrating less rigorous nutritional requirements. Neviati et al. (2009) noted that these microorganisms were not cultivated in MRS or WAM when they are the minority of a microbial population, but they can grow in CAM. When they become the dominant population in the absence of high nutritionally demanding competitive microorganisms, presumably autolysed, then they can be detected in MRS or WAM (Neviani et al. 2009). This study showed that thermophilic SLAB dominate at the start of ripening due to the temperature conditions during production, but that mesophilic NSLAB increase during the later stages of cheese ripening due to lower temperatures and their ability to metabolize alternative substrates in the absence of lactose. Decreases in the number of bacteria due to autolysis were related to increased peptidolytic activity in the cheese.

Temmerman et al. (2004) have already reviewed the application of phenotypic and genotypic techniques that have been proven to be useful for the identification and typing of LAB. For strain typing of LAB, phenotypic tests provide evidence of the metabolic capabilities of strains, whereas the advantages of genotyping include the stability of genomic DNA as its composition is independent of culture conditions or preparation methods according to Fitzsimons et al. (1999). Both types of techniques may exhibit several weaknesses for identification and typing such as poor reproducibility, ambiguity of some techniques, extensive logistics for large-scale investigations, and poor discriminatory power for phenotypic techniques. Although genotypic techniques provide a more robust classification and differentiation, such as restriction fragment length polymorphism (RFLP), ribotyping, pulsed-field gel electrophoresis (PFGE), amplified ribosomal DNA restriction analysis (ARDRA), and repetitive extragenic palindromic PCR, some limitations exist (cost, equipment, and databases). In addition, some of the

molecular techniques applied to the intraspecific typing of different LAB strains such as randomly amplified polymorphic DNA (RAPD) are not highly comparable among laboratories. Finally, all of them have to be applied to pure cultures of microorganisms (Martin-Platero et al. 2009). The importance of combining phenotypic and genotypic methodology is especially evident for discriminating within the *Lactococcus lactis* species as strains within the *cremoris* genotype may have the *lactis* phenotype (Kelly et al. 2010). In conclusion, a polyphasic or combined approach should be applied to obtain more complete and realistic information about the microbial communities of cheese (Ercolini et al. 2001; Martin-Platero et al. 2009; Temmerman et al. 2004).

2.1.1 Sequence-based methods for characterizing cultured isolates

The sequencing of 16S rRNA is the most widely used technique for identifying bacterial species isolated from cheese (Randazzo et al. 2009; Temmerman et al. 2004). Yeasts isolated from cheese can be identified by sequencing of the D1/D2 region of the 26/28S rRNA gene (Kurtzman and Robnett 1997; Mounier et al. 2009). For the identification of genus or species, the partial or complete sequence of these genes is compared with sequences from known microorganisms by the aid of specialized software programs and/or online tools for determining the closest sequence (Giraffa and Carminati 2008).

The differentiation of LAB using protein-coding genes has been previously explored in order to select the most appropriate genes for bacterial taxonomy and phylogeny (Coppola et al. 2008). Palys et al. (1997) showed that the DNA sequences of protein-coding genes are more effective than 16S rRNA for bacterial classification for distinguishing very closely related species. This limitation in resolving power and the variation in sequence among multiple copies of the gene lead to over- and underrepresentation of some bacterial species when using 16S rRNA genes as targets. Coppola et al. (2008) reviewed the use of single-copy target genes showing sequence differences sufficient for improving phylogenetic resolution within species. Since that review, these and other target genes have proven useful, such as *tuf* for *Propionibacterium* (Falentin et al. 2010b), *rpoB* (Martin-Platero et al. 2009), and *pheS* (phenylalanyl-tRNA synthase) for *Lactobacillus paracasei* (Van Hoorde et al. 2010a; Van Hoorde et al. 2010b). In Gouda cheese, isolates that could not be identified by $(GTG)_5$-PCR typing were distinguished by *pheS* gene sequencing, thus emphasizing the importance of a polyphasic approach with molecular methods as well. For Gouda cheese, discriminant analysis of a composite data set of community profiling showed that while cheeses could not be distinguished by ripening time (8 versus 12 weeks), different cheese types could be discerned and the observed batch-to-batch variation was most probably due to the milk starting material. In a further study, the prevalence of specific *L. paracasei* adjunct cultures was shown throughout the ripening phase, and each could be associated with a particular volatile compound profile corresponding to either proteolyic or lipolytic activities (Van Hoorde et al. 2010b).

DNA sequence analysis of a specific subset of conserved genes is called multilocus sequence analysis (MLSA) and multilocus sequence typing (MLST) for

DNA sequencing of internal fragments of at least seven housekeeping or protein-coding genes for a given species (Maiden et al. 1998). The selected genes must show a higher degree of variability between related bacteria than the 16S rRNA gene in order to distinguish among subspecies or strains (Sarmiento-Rubiano et al. 2010). The concatenated sequences of the selected genes can be analyzed for nucleotide similarity, or the polymorphisms can be assigned as alleles in order to designate sequence types from allelic profiles. MLSA has been shown to be a powerful technique for diversity analysis within related species of the *Lactobacillus acidophilus* group or among highly related strains of lactococci (Rademaker et al. 2008; Taïbi et al. 2010) as well as for other dairy microorganisms, as reviewed by Coppola et al. (2008). Although MLST studies have not been applied to determine the population structure or the diversity of isolates within a given cheese sample, this approach provides unambiguous results that are directly comparable between laboratories (Cai et al. 2007; Coppola et al. 2008; Dalmasso et al. 2011; de las Rivas et al. 2006; Diancourt et al. 2007; Maiden et al. 1998). MLSA data will also help in selecting genes with higher resolving power for microbial community profiling techniques.

As a consequence of the development of sequencing technology, many projects of whole-genome sequencing of both eukaryote and prokaryote micro-organisms have been carried out in the past years. The genomes of many dairy and probiotic microorganisms have been sequenced: *L. lactis* subsp. *lactis* IL1403 (Bolotin et al. 2001) and KF147 (Siezen et al. 2010), *L. lactis* subsp. *cremoris* MG1363 (Linares et al. 2010; Wegmann et al. 2007), SK11 (Makarova et al. 2006) and NZ9000 (Linares et al. 2010), *L. acidophilus* NCFM (Altermann et al. 2005), *Lactobacillus casei*, *Streptococcus thermophilus*, *Pediococcus pentosaceus* (Makarova et al. 2006), and *Propionibacterium freudenreichii* CIRM-BIA1 (Falentin et al. 2010a), to name only a few. The Genomes Online Database contains a more complete listing of sequencing projects to date, either in progress or completed (http://www.genomesonline.org/). The availability of these complete genomic sequences enables genome-wide comparison between and within subspecies with DNA microarray-based comparative genomic hybridization (CGH). An oligonucleotide array targeting five *L. lactis* genomes has been used to distinguish three subspecies among 39 strains of *L. lactis*, which correlated with the dairy or plant ecological niche (Bayjanov et al. 2009). Within *L. lactis* subsp. *cremoris*, a targeted oligonucleotide microarray based on the genome of SK11 revealed the presence or divergence of genes involved in sugar metabolism, amino acid biosynthesis, osmoregulation, and proteolytic activity among eight strains (Taïbi et al. 2010). It was concluded that the combination of information obtained by CGH and MLSA for strain classification opens new opportunities for industrial applications based on the selection of strains according to their genetic profiles (Taïbi et al. 2010). Tan-a-Ram et al. (2011) combined phenotyping with genotyping and transcriptome analysis for studying the diversity of six strains of *L. lactis* subsp. *lactis*. Although a core genome of 99% of the IL1403 ORFs spotted on the microarray was found, there was variation in acidification rates among strains, and considerable diversity in transcriptomic profiles was observed. The authors suggest that regulatory networks may be strain-specific as 37 regulatory genes showed differences in expression level among the six strains.

The application of these methods has underscored the importance of strain selection to control the effect of diversity on product quality, but the culturable microorganisms underestimate the true diversity of the cheese microbiota.

2.2 Culture-independent methods

In contrast to culture-dependent techniques, culture-independent methods are those which do not require culturing the microorganisms (b and c in Fig. 1). This approach was applied to food systems for the first time by Ampe et al. (1999) to provide a more realistic view of microbial diversity in a fermented maize dough called Mexican Pozol. As Pozol dough is shaped into balls, the spatial distribution of the microbiota is influenced by the presence of gradients from the inside to the periphery. In this study, the use of RNA probes and denaturing gradient gel electrophoresis (DGGE) analysis as molecular tools targeted streptococci and lactobacilli strains showing that *Lactobacillus fermentum* and *Lactobacillus plantarum* are the dominant species in Pozol fermentation. Ampe et al. (1999) concluded that a polyphasic approach is also applicable to other fermented foods, such as cheese. Coppola et al. (2001) and Ercolini et al. (2001, 2003b, 2004) were among the first to successfully apply these methods to Italian Mozzarella and Stilton cheese in order to better understand the dynamic changes and diversity of microbial communities and determine the dominant species and variability of such foods (Randazzo et al. 2002). They could distinguish traditional cheeses from industrial cheeses by their higher diversity (Coppola et al. 2001). In fact, cheeses made without a starter culture (natural inoculation) showed the highest diversity compared with cheeses inoculated with milk, whey, or industrial starters. The diversity of natural whey cultures was also examined, showing the bias of culture methods in underrepresenting the number of species (Ercolini et al. 2001). Furthermore, microbial succession was followed throughout the Mozzarella cheese-making process, showing the dominance of the natural whey starter culture in the curd, while the diversity of the raw milk microbiota diminished in the curd due to competition with the starter and the processing conditions (hot stretching, for example; Ercolini et al. 2004). The natural whey starter composition was also related to geographical origin (Mauriello et al. 2003). Analysis of Stilton cheese revealed microenvironments in the spatial distribution of bacteria according to pH and oxygen gradients as well as, presumably, nutrient gradients in the core compared to the veins and crust (Ercolini et al. 2003b).

In food matrices such as cheese, culture-independent methods have rapidly been recognized as a valuable approach for the study of biodiversity and identification of microbial species (Ercolini et al. 2001). Briefly, these methods are based on the direct extraction of total DNA or RNA from the cheese matrix. Then, the DNA or cDNA (after reverse transcription of the total RNA) is amplified by PCR and the resulting samples are cloned and sequenced to construct a library for species identification (b and c in Fig. 1). Very complete papers have reported protocols (b in Fig. 1) to extract DNA directly from dairy products for further molecular investigation of cheese structure and dynamics (Cocolin et al. 2002; Coppola et al. 2001; Ogier et al. 2004; Randazzo et al. 2002). These protocols are now well established and routinely optimized in dairy research laboratories in order to apply

molecular methods to identify microorganisms in substrates. In addition, many protocols are available to focus on RNA extraction (c in Fig. 1) directly from the dairy matrix to facilitate the investigation of microbial activities (Bonaïti et al. 2006; Monnet et al. 2008; Randazzo et al. 2002; Ulve et al. 2008). Since previous reviews were published, those using a mechanical bead-beating procedure to lyse the bacterial cells in order to ensure efficient extraction of nucleic acids have gained popularity (Randazzo et al. 2002; Ulve et al. 2008).

This section provides an overview of the principal culture-independent methods used to study the complex cheese microbial ecosystem. By targeting the DNA and or RNA directly extracted from cheese matrix as mentioned above, molecular techniques can estimate both microbial diversity and activity, respectively (Table 1 and Fig. 1). At this stage, it may be possible to detect microbial communities and characterize their metabolic activity for better predictions in terms of cheese quality, safety, and acceptability that are expected during cheese making, as expertly reviewed by Coppola et al. (2008). Notably, Coppola et al. (2008) introduced the possibility of monitoring not only the identity of microorganisms ("Who is there?") but also their in situ metabolic activities ("Who is doing what?") during dairy fermentations. Therefore, advances in these two directions are emphasized here.

2.2.1 Microbial diversity

Final cheese quality is dependent on the contribution of primary, secondary, and adventitious microbiota. Therefore, the application of molecular culture-independent tools interest food microbiologists in order to: directly identify members of a community and to assess their abundance; to reliably fingerprint complex bacterial communities; to analyze the diversity and dynamics of the dominant microbial community; to compare the spatial and temporal changes in bacterial community structure; and to accurately quantify target species (Jany and Barbier 2008).

2.3 Direct identification

Direct identification and the study of the dynamics and diversity of microorganisms during cheese ripening and in the final cheese product can benefit from reliable PCR-based tools such as PCR-DGGE, PCR temperature gradient gel electrophoresis (TGGE), single-strand conformation polymorphism PCR (SSCP-PCR), terminal restriction fragment length polymorphism (T-RFLP), and length heterogeneity PCR (LH-PCR), or automated ribosomal intergenic spacer analysis (ARISA) techniques (Coppola et al. 2008).

Sequence analysis of 16S rRNA gene-based clone libraries is a promising tool to achieve the goal of identifying key microorganisms in the curd and cheese samples without the limitations imposed by culture-dependent methods and biochemical identification (Rasolofo et al. 2010). PCR-amplified sequences can be directly cloned and sequenced for species identification, composition, or structure of the community members (Delbes et al. 2007; Duthoit et al. 2003; Randazzo et al. 2002). The prokaryotic small subunit rRNA gene, commonly called the 16S rRNA gene, is the most frequently used molecular marker in microbial ecology because of its characteristics such as universal abundance,

Table 1 Culture-independent methods widely applied to cheeses for genomic fingerprinting of microbial diversity

Techniques	Applications to cheese samples	References
PCR-DGGE (TGGE)	Artisanal fresh sheep cheese (Croatia)	Pogačić et al. (2010b)
	Danish raw milk cheese	Masoud et al. (2010)
	Types of Gouda cheese (Belgium)	Van Hoorde et al. (2010a); Van Hoorde et al. (2008)
	Artisanal Pecorino Crotonese cheeses (Italy)	Randazzo et al. (2010); Randazzo et al. (2006)
	Surface of five traditional Italian cheeses	Fontana et al. (2010)
	Three batches of Castelmagno PDO cheese (Italy)	Dolci et al. (2010); Dolci et al. (2008)
	Two batches of a blue-veined cheese (Spain)	Alegria et al. (2011)
	Istrian cheese (Croatia)	Fuka et al. (2010)
	Five artisanal cheese samples (Slovenia)	Lorbeg et al. (2009)
	Fontina PDO cheese (Italy)	Dolci et al. (2009)
	Gorgonzola rinds and maturing shelf (Italy)	Cocolin et al. (2009)
	Calenzana, Corsican raw milk (goat and sheep) cheese	Casalta et al. (2009)
	Traditional Spanish Casin cheese	Alegria et al. (2009)
	Feta cheeses produced by different Greek manufacturers	Rantsiou et al. (2008b)
	12 months ripened Parmigiano Reggiano cheeses (Italy)	Gala et al. (2008)
	"Robiola di Roccaverano" PDO cheese (Italy)	Bonetta et al. (2008)
	Quesailla Arochena and Torta Arochena cheese (Spain)	Martin-Platero et al. (2009)
	Egyptian Domiati cheese	El-Baradei et al. (2007)
	Tina wood vats used in the Ragusano cheese	Licitra et al. (2007)
	Spanish, blue-veined Cabrales cheese	Florez and Mayo (2006a); Florez and Mayo (2006b)
	Swiss-type, semi-hard and red-smear cheeses (France)	Ogier et al. (2004)
	Stilton cheese (UK)	Ercolini et al. (2003b)
	Sicilian Ragusano cheese (Italy)	Randazzo et al. (2002)
	Mozzarella cheese (Italy)	Ercolini et al. (2004); Ercolini et al. (2001)
SSCP	Livarot cheese (France)	Mounier et al. (2009)
	Saint-Nectaire-type technology cheese (France)	Saubusse et al. (2007)
	Two types of French red-smear cheese (France)	Feurer et al. (2004a)
	Salers cheese (France)	Callon et al. (2006); Duthoit et al. (2005); Duthoit et al. (2003)
	Semi-hard cheese (France)	Delbès and Montel (2005)
	Farm productions of Saint-Nectaire cheese (France)	Delbès et al. (2007)
T-RFLP	Camembert cheese (Canada)	Arteau et al. (2010)
	Smear-ripened Tilsit cheese (the Netherlands)	Rademaker et al. (2005)
LH-PCR	Grana Padano cheese (Italy)	Gatti et al. (2008)
	Spanish farmhouse cheese (Spain)	Martin-Platero et al. (2009)
ARISA	Camembert cheese (Canada)	Arteau et al. (2010)

evolutionary and phylogenetic properties, high discriminatory potential, multiple-copy nature enabling sensitive analyses, and availability of sequences in public databases. Genetic targets should have both variable and conserved regions. Variable regions, which allow discrimination of taxonomic levels, are flanked by conserved sequences representing the annealing sites for universal PCR primers. Sequence analysis of 16S rRNA gene-based clone libraries has been used in different studies for the identification, diversity, and dynamics of the bacterial community in cheese (Feurer et al. 2004a; Randazzo et al. 2002). Microbial community composition during the manufacture and ripening of protected denomination of origin (PDO) cheese samples made with natural thermophilic culture was obtained with 16S rRNA clone library sequencing data obtained from both DNA and reverse-transcribed RNA (cDNA) amplification (Carraro et al. 2011). This very recent study on Montasio cheese shows that biases due to nucleic acid extraction or amplification can be minimized, but a large number of clones should be analyzed when there are predominant species present. In order to compensate this limitation, qPCR was used to demonstrate the impact of thermization on reducing *Pseudomonas* spp., but this group was still active in terms of cDNA detection until the ripening phase (Coppola et al. 2008). Bacterial community dynamics observed by culture-dependent methods are enhanced by the analysis of 16S rRNA gene clone libraries (Rasolofo et al. 2010). More than 200 operational taxonomic units were found in untreated and treated milk samples stored at 4 °C (Rasolofo et al. 2010).

Cloning and sequencing provide a great detail of phylogenetic information along with quantification of the relative abundance of clones within a microbiota sample. The limitation of the use of clone libraries is that, depending on the sample to be analyzed, the number of clones to be sequenced can attain thousands of clones in order to cover the phylogenetic richness in the clone library (Rasolofo et al. 2010). However, coverage of clone libraries can be estimated with programs such as DOTUR (http://schloss.micro.umass.edu/software/dotur.html) or ESTIMATES (http://viceroy.eeb.uconn.edu/estimates).

The cloning and sequencing approach is time-consuming and expensive. In the presence of dominant species, the number of clones to be analyzed in order to obtain an accurate and realistic view of the microbial community should be several thousand clones (Carraro et al. 2011). Hence, next-generation sequencing or high-throughput sequencing methods such as pyrosequencing have been developed (Margulies et al. 2005) that could replace cloning steps. Humblot and Guyot (2009) have for the first time applied this technology to a cereal fermented food, offering many opportunities of rapid genomic analysis of other food samples such as spinach (Lopez Velasco et al. 2011) or fermented foods (Park et al. 2011).

2.4 Fingerprinting complex bacterial communities

Justé et al. (2008), Jany and Barbier (2008), Pogačić et al. (2010a), and Randazzo et al. (2009) amply reviewed fingerprinting methods such as DGGE, TGGE, SSCP, T-RFLP, and ARISA to generate a general view of the microbial community of cheese matrices and food processes. The outline of these methods will be concisely

described for facilitating the interpretation of "Reverse transcription PCR-based analysis" in Section 2.6.

A considerable number of papers (Table 1) report the use of PCR temperature or denaturing gradient gel electrophoresis (T/DGGE) as fingerprinting methods for studying bacterial diversity and population dynamics during cheese manufacturing (Coppola et al. 2008; Ercolini 2004) by using total DNA in PCR amplification (Muyzer et al. 1993) or the metabolically active populations by using the total RNA (Dolci et al. 2010; Randazzo et al. 2002; Rantsiou et al. 2008b). Ercolini (2004) was the first to have reviewed the microbiological goals of DGGE fingerprinting, which is more precisely to achieve a culture-independent assessment of the microbiota of food products. The presence of heterogeneous copies of rDNA operons harbored by some microorganisms is one of the limiting factors of the T/DGGE technique which results in the detection of several bands for a single bacterial or fungal species (Florez and Mayo 2006b; Randazzo et al. 2010). In addition, related species with the same V3 sequences and those with different V3 sequences of the 16S rRNA but having the same T_m migrate at the same position (Duquenne et al. 2010; Larpin et al. 2006). Other phylogenetic markers such as the β-subunit of the RNA polymerase (*rpoB*) gene are used as an alternative to the 16S rRNA gene to overcome limitations of ribosomal gene markers which often present intraspecies heterogeneity and multiple copies (Martin-Platero et al. 2009; Rantsiou et al. 2004). Some species revealed using *rpoB* were not detected using the V3 region of the 16S rRNA gene with Spanish goats' milk cheese (Martin-Platero et al. 2009). However, the 16S rRNA gene gave greater species richness but less diversity than the *rpoB* gene. Molecular methods complemented culture-dependent methods in this case, revealing different species, although species diversity was greater by genotyping cultured isolates compared with direct molecular profiling by PCR-TGGE. This shows the importance of gene selection as it determines the limits of the resolving power of the culture-independent method. An important result from this study is the demonstration of competition during amplification for some species, but not others. Thus, *L. lactis* and *L. plantarum* interfered with the detection of *L. paracasei*. This could explain why *L. paracasei* was not consistently found in Gouda cheese by DGGE on total DNA extracts, but could be detected in DNA extracted from culture-enriched samples where competition was presumably lower (Van Hoorde et al. 2010b). Future work should be done to select primers and or genes that reduce this species bias.

Finally, the fragments to be resolved by T/DGGE are limited to 500 bp, which represents a limiting factor for sequence analysis and, eventually, probe design (Ercolini 2004). PCR-DGGE is not a quantitative method; band intensity was not always in agreement with plate counts. In a study on raw milk and fresh curds of Fontina cheese (Giannino et al. 2009), the limits of the PCR-DGGE technique were made evident. Particularly, the species *L. lactis* and *Leuconostoc lactis* were present at very low levels in curd samples (1/10), while they were frequent in milk. The formation of a compact coagulum after heating at 47–48 °C leads to a concentration of other leading species, such as streptococci and enterococci; hence, lactococci and leuconostoc became a minor group that could not be revealed by PCR-DGGE, indicating that this method is not well adapted to detect subdominant populations. Differential lysis of the microbial populations, presence of amplifiable DNA from

dead microorganisms, and detection of non-viable cells can bias DGGE results, and in fact all PCR-based methods (Alegria et al. 2009).

Furthermore, TGGE and DGGE should be combined to provide a higher resolution profiling of bacteria comprising the ecosystems of cheeses in order to compensate the limitation of TGGE in poor resolution of species with high G+C content genomes (Ogier et al. 2004). An original application of DGGE (or TGGE) is the identification of food products depending on their geographical origin. Giraffa and Neviani (2001) introduced and defined the term of bacterial biogeography to describe bacteria that are endemic to a specific area or host. By comparing profiles from DGGE from different farmers, Randazzo et al. (2010) noted that each farmhouse or manufacturing facility may be characterized by an independent microbial population. However, DGGE fingerprinting did not reveal significant differences between Gouda cheeses produced at two locations (Van Hoorde et al. 2010a). The limit of detection of minority species may explain these contradictory results because species representing <1% of the total community would not be detected in the T/DGGE gel (Muyzer et al. 1993; Ogier et al. 2004). The sensitivity of DGGE for the analysis of cheese is estimated to be 10^4 CFU.g^{-1} (Cocolin et al. 2004). In addition, similar bacterial taxa could be present at the two production facilities, but may be represented by different groups of strains that were not differentiated by T/DGGE (Van Hoorde et al. 2010a). Thus, techniques focused on intraspecies diversity would contribute complementary information.

Single-strand conformation polymorphism (SSCP) is similar to PCR-D/TGGE as it allows the separation of different DNA fragments of similar length, also relying on electrophoretic separation of PCR products. SSCP has been applied in several instances to analyze mutation or dynamics between microbial populations at the genus and species levels in cheese (Table 1; Callon et al. 2006; Delbes et al. 2007; Delbes and Montel 2005; Duthoit et al. 2003; Feurer et al. 2004b; Mounier et al. 2009; Saubusse et al. 2007). The main target probes of SSCP are the V2 and V3 regions of the 16S rRNA gene for bacteria and the 18S (Callon et al. 2006) or 26/28S rDNA gene for yeasts (Feurer et al. 2004a). SSCP has been used to profile the yeast community in Livarot (Mounier et al. 2009) and Salers cheese (Callon et al. 2006). According to Feurer et al. (2004a), SSCP analysis allowed them to detect the presence of unknown species corresponding to non-assigned peaks that were not identified either by the cultivation technique or by the clone sequencing strategy. However, it was not possible to identify them precisely, even at the family or genus level, thus constituting a limitation of the SSCP technique. Feurer et al. (2004a) concluded that the SSCP technique gives a general overview of the relative abundance of the dominant members of the surface flora and their evolution during cheese production.

T-RFLP is increasingly used to analyze microbial communities due to its simplicity and reliability for bacterial 16S rRNA genes (Sánchez et al. 2006; Schütte et al. 2008). The main advantages of the T-RFLP technique in comparison to other culture-independent approaches such as T/DGGE are high throughput and high sensitivity without the need for direct sequence information for presumptively identifying and monitoring specific bacterial populations within a microbial community. However, misidentification may result from the fact that some discrepancies may appear during assignment of the peaks in T-RFLP profiles with

regard to the migration of reference clones or web databases (Dunbar et al. 2001; Nocker et al. 2007; Schütte et al. 2008) as well as from the presence of pseudo T-RF which may be formed during PCR (Egert and Friedrich 2003). T-RFLP has become a valuable method for rapidly comparing the temporal changes and relationships between bacterial communities, revealing the most dominant sequences in dairy samples. T-RFLP and PCR-DGGE were used to study the dynamic of milk microbiota, showing that the diversity of the bacterial community was strongly affected by milk treatments such as the addition of CO_2, thermization, or microfiltration (Rasolofo et al. 2011). The microbial community structure changed continuously during storage of raw and treated milk samples at low temperatures. Moreover, T-RFLP showed a higher resolution than PCR-DGGE. Particularly, PCR-DGGE gels allowed only the detection of bands representing dominant populations. Thus, T-RFLP is an efficient method for the comprehensive analysis of milk microbiota during cold storage.

Automated ribosomal intergenic spacer analysis (ARISA), perhaps due to its similarity with T-RFLP and D/TGGE, has not yet been widely used for microbial community analysis of food samples (Juste et al. 2008). A profiling approach based on T-RFLP and ARISA was used for the first time in combination with principal component analysis to assess fungal changes in Camembert cheese according to processing parameters (Arteau et al. 2010). This approach was sufficiently sensitive to detect differences in fungal populations between batches and types of Camembert or Brie, suggesting that T-RFLP and ARISA may be applied for the early detection of fungal contaminants.

LH-PCR analysis is similar to the T-RFLP method in that T-RFLP identifies PCR fragment length variations based on restriction site variability, whereas LH-PCR analysis distinguishes different organisms based on natural variations in the length of sequences of the 16S rRNA gene (or other genes; Giraffa and Neviani 2001). This technique provides a quick and easy way to evaluate technological functionality of many different whey starter cultures on the basis of qualitative and quantitative variations of the dominant LAB species, although LH-PCR is subject to certain biases inherent to the PCR process (Lazzi et al. 2004). Length heterogeneity reverse transcriptase PCR has also been applied to study the metabolically active microbial populations recoverable from Grana Padano cheese whey starters (Fornasari et al. 2006; Rossetti et al. 2008; Santarelli et al. 2008). The limit of sensitivity (10^5 CFU.mL^{-1}) of *S. thermophilus* in eight whey starters of Grana Padano cheese suggests a need to combine LH-PCR with traditional cultivation methods (Rossetti et al. 2008). As demonstrated during ripening of Parmigiano Reggiano, the low sensitivity of LH-PCR cannot provide a quantitative analysis but a means to follow the dynamics of whole and lysed bacterial cells (Gatti et al. 2008).

2.5 Monitoring specific species in situ

There is considerable interest for methods allowing in situ detection of microorganisms within the cheese matrix (Jeanson et al. 2011). Even though some molecular techniques have great potential for studying the spatial distribution of microbial populations in foods, few allow the manipulation of cryosections in

situ in the food matrix, such as cheese, without alteration. For this purpose, Ercolini et al. (2003a) were the first to apply an accurate and reliable technique, fluorescence in situ hybridization (FISH), to study the microbial communities in intact food, allowing the specific location of bacteria in the cheese matrix. FISH is a non-PCR-based molecular tool that employs ribosomal RNA-targeted oligonucleotide probes to rapidly and reliably detect and enumerate specific microorganisms by fluorescent microscopy (Bottari et al. 2006). Bottari et al. (2006) have reviewed the methodological aspects of FISH technology for microbiology analysis. So far, Cocolin et al. (2007) have optimized a FISH protocol for cheese samples as a direct in situ analysis with fluorescently labeled probes in complex samples requiring a highly efficient sample pretreatment. They pointed out that the detection limit of the FISH technique is about 10^3 CFU.mL^{-1}. Rantsiou et al. (2008b) studied the viable cells in Feta cheese by FISH analysis, which corresponded with results obtained by PCR-DGGE on the RNA. The presence of thermophilic *Lactobacillus* strains observed by FISH was not detected at the same time by plating methods. FISH has also been applied for the characterization of the yeasts and bacteria found at the surface of smear cheeses, confirming that *Gamma-Proteobacteria*, *Candida catenulate*, and *Geotrichum* sp. were the most abundant species (Mounier et al. 2009). Hence, with the FISH technique, the knowledge of composition and distribution of microorganisms in the cheese matrix can be useful for safety and technological aspects in dairy industries.

Two distinct applications for monitoring specific microbial community members are quantitative PCR (qPCR) and reverse transcription quantitative PCR (RT-qPCR). The qPCR technique is used especially in studies of DNA, while RT-qPCR is devoted to the study of specific expressed genes (mRNA), which will be discussed in the next section. The qPCR technology, based on the detection and quantification of a fluorescent marker, either a specific probe or a DNA-binding agent such as SYBR green, is a useful tool for effective detection, identification, and quantification of bacteria in different types of samples or products. In addition, on the basis of sensitivity and precision, qPCR can solidly support the accuracy of the data obtained by methods such as T/DGGE or T-RFLP (Rasolofo et al. 2010). The qPCR has several additional advantages such as speed and reproducibility and does not require large quantities of sample. However, the quantification limit has been estimated at 10^3–10^4 CFU.g^{-1} in cheese (Rantsiou et al. 2008a). The distinction between viable and non-viable bacteria brings a major challenge for the monitoring of microbial populations as DNA could be present for quite some time after cell death (Sánchez et al. 2006). The quantitative analysis of total DNA can lead to a substantial overstatement of the presence of viable microorganisms. The lack of differentiation between the DNA of viable and non-viable bacterial cells is a significant barrier to the use of qPCR (Nocker et al. 2006) that can be resolved by eliminating the amplification of DNA from dead cells, for example by complexing with agents such as propidium monoazide (see more on this in "Reverse transcription PCR-based analysis" in Section 2.6).

The qPCR approach has also been used to study the community dynamics of yeasts of technological interest in cheese by Larpin et al. (2006). Specific primers were able to detect and quantify *Geotrichum candidum* as a major dominant

species throughout the ripening process, while *Yarrowia lipolytica* appeared at the end of Livarot cheese ripening. Despite the difficulty of obtaining a good nucleic acid extract from the surface of Livarot cheese, a robust quantitative method was successfully developed by these authors to target dominant and subdominant species as well as their dynamics on red-smear Livarot cheese.

2.5.1 Microbial activity

Despite the availability of genome sequences for some LAB (Makarova et al. 2006), our understanding of the activities and physiological functions of the cheese microbiota requires improvement. The microbial ecology and dynamics of cheese is characterized by the presence of metabolically active population that is not suitably detected by culture-dependent methods. Classical cultivation methods cannot give complete information on microbial diversity in cheese. Moreover, bacteria may remain metabolically active, but not necessarily be able to divide and thus are not detected as cultivable microorganisms (Ganesan et al. 2007). Furthermore, other bacteria might be uncultivable mainly on the rind of surface-ripened cheese where adverse conditions such as nutrient depletion, low temperature, and other stresses can sub-lethally damage microorganisms (Giraffa 2004; Giraffa and Neviani 2001). These authors distinguished the uncultivable or viable but non-cultivable (VBNC) bacteria from the sub-lethally injured cells which are those that need plating on non-selective media to grow and form colonies. In the cheese ecosystem, the dynamics of growth, survival, and biochemical activity of microorganisms should be understood via a combination of molecular tools, while traditional cultivation methods have a role in physiological characterization and may contribute to enriching subdominant populations. In addition, the relatively recent introduction of molecular techniques to quantify the expression of genes from complex environmental samples initiated a greater understanding of the roles and diversity of bacterial populations.

2.6 Reverse transcription PCR-based analyses

In recent years, dairy microbiologists have understood the usefulness of reverse transcription PCR (RT-PCR) to target the RNA molecules in order to characterize microorganisms and metabolic activities that are expected during the most important phases of cheese making. By reverse transcription of RNA molecules, the expression of transcribed genes and their activities can be evaluated. Therefore, coupled with the methods described above for microbial detection, reverse transcription PCR-based techniques make it hopefully possible to understand which of the important activities are being carried out in each phase of cheese production and ripening. In the case of cheese, such techniques have been coupled with other molecular tools on several occasions to monitor microbial activity. Duthoit et al. (2005) showed interesting results on the active microbiota of Salers cheese by using RNA as a template in the RT-PCR-SSCP method. These authors have compared DNA and RNA SSCP profiles and revealed the differences in microbial composition of Salers cheese. Both methods allowed a global view of the dynamics of the bacterial community which was insufficient in explaining the diversity of the sensorial qualities of R.D.O. Salers cheese. The authors suggested that rapid methods to

measure genomic microbial diversity and specific activities as well as aromatic compound diversity should be developed to overcome this aspect (Duthoit et al. 2005).

Sanchez et al. (2006) used an RT-PCR-based T-RFLP method for a semi-quantitative analysis of the metabolically active populations in defined strain cultures of *L. lactis* subsp. *lactis* and *Leuconostoc citreum*, two mesophilic LAB species routinely used in cheese manufacture. When cultivated together, one strain of *L. citreum* was maintained at about 20% of the total culture, while the second species gradually decreased over the 24-h milk fermentation. The study showed a larger proportion of *L. lactis* subsp. *lactis* quantified by T-RFLP at 24 h of incubation than that measured by viable counts. This was explained as the result of the detection of VBNC microorganisms, demonstrating the usefulness and reliability of RT-PCR-based T-RFLP method for monitoring the population dynamics of the metabolically active fraction of defined dairy starters, which avoids overestimation of the dominant population that can occur in the presence of non-viable cells (Sánchez et al. 2006).

More recently, Rossetti et al. (2008) have obtained interesting results demonstrating the usefulness of combining RT-PCR and LH-PCR methods to describe the metabolically active LAB in Grana Padano cheese that were not detected by Lazzi et al. (2004). As active bacteria have a higher number of ribosomes than dead cells, this approach gave an estimate of metabolically active LAB populations present in the whey starters (Fornasari et al. 2006).

RT-PCR-DGGE has also been performed to reveal metabolically active populations in different types of cheese (Dolci et al. 2010; Randazzo et al. 2002; Rantsiou et al. 2008b). In Feta cheese samples, comparison of the DNA and RNA analyses indicated the presence of VBNC populations of *S. thermophilus* and *Lactococcus* spp. (Rantsiou et al. 2008b). *S. thermophilus* was detected by RT-PCR-DGGE and FISH after several months of storage in brine (Rantsiou et al. 2008b). Lactococci have also been detected by both PCR-DGGE and RT-PCR-DGGE in Castelmagno PDO production, showing an active involvement during both manufacturing and ripening (Dolci et al. 2010). An active presence of *L. lactis* throughout cheese ripening has been hypothesized by these authors, although lactococci decreased during ripening according to the cultivation methods. These results underline possible contributions of live starter cultures during ripening to the characteristics of the final product (Masoud et al. 2011).

For quantifying the relative abundance of viable bacteria, a promising strategy is the use of a DNA intercalating dye, ethidium monoazide bromide (EMA) or propidium monoazide (PMA; Juste et al. 2008; Nocker et al. 2006). These dyes penetrate only into the non-viable cells whose membrane integrity is compromised and covalently bind to the DNA by photo-activation. The addition of EMA or PMA to food samples, followed by PCR analysis, has been able to differentiate between genes in viable and non-viable cells (Nocker et al. 2006). Therefore, the microbial community treated by EMA or PMA should closely represent the active population. To our knowledge, the application of PMA and qPCR for the quantification in cheese of viable strains has not been reported yet, but this procedure was successfully used for quantification of *L. acidophilus*, *L. casei* and *Bifidobacterium animalis* subsp. *lactis* in mixed cultures with yoghurt starter species. The main

advantages are that this method allows an easy, simple, and precise quantification of viable bacteria within a detection range of 5 log units (García-Cayuela et al. 2009).

The detection of RNA by reverse transcription in RT-qPCR, as opposed to DNA, is considered as a better indicator of cell activity. Nevertheless, working with RNA is technically a greater challenge because RNA is easily degraded, which can lead to problems of reproducibility, requiring rigorous standardization of methods and normalization of results. In addition, the level of expression of RNA depends on the physiological status of the cell, making accurate measurements of the number of bacteria more difficult.

Moreover, the dynamics and activity of microbial communities during processing, production, storage, and even consumption of a functional food may be followed by RT-PCR using specific primers based on the 16S rRNA gene or another target gene (Falentin et al. 2010b). From metabolic characteristics and sequences of each strain, it is possible to design primers and probes useful in determining the abundance and the relative activity of each strain during manufacture, ripening, and storage of cheese. Quantification of nucleic acids could be determined either in absolute terms by calibration with a standard or relative to a reference gene. For absolute quantification, calibration requires measurement for different concentrations of the target gene or transcript. This indicates a curve of the accumulation of fluorescence at a specific point of amplification (threshold cycle) versus the amount of nucleic acid and a measure of the amplification efficiency (which should be close to 2). The calibration curve makes it possible to determine the concentration via a measure of a threshold cycle of the fluorescence accumulation. For relative quantification, reference and target genes are measured together to compare their concentration. The 16S rRNA sequence is useful to develop specific methods for the quantification of viable cells by RT-qPCR. This method allows both the absolute quantification of cells and relative quantification of a target gene expression (or transcript) compared with that of a reference gene. Recent important findings about the use of RT-qPCR on Camembert cheese contaminated by *Staphylococcus aureus* have been carried out by Ablain et al. (2009). Transcriptomics analysis from this study revealed the suitability of RT-qPCR to accurately identify the *S. aureus* contaminants in Camembert cheese. Rantsiou et al. (2008a) selected the intergenic spacer region between the 16S and 23S rRNA gene to develop a quantitative test to detect *Listeria monocytogenes* using DNA and RNA extracted from several food matrices including cheese, which gave a quantification limit of 10^4–10^5 CFU.g^{-1}. Falentin et al. (2010b) used the RT-qPCR technique to track the metabolic activity of specific prokaryotic strains throughout the different phases of Emmental cheese manufacture and ripening. With the combination of qPCR and RT-qPCR, they have been able to monitor microbial growth and activity of *P. freudenreichii* and *L. paracasei* at 12 steps of ripening of Emmental cheese. In particular, lowered expression of the *tuf* gene could be associated with reduced metabolic activity, while increased expression of *gro*L was linked to stress response during ripening in the cold room. Results from qPCR were consistent with viable counts to enumerate bacterial populations, and the reliability of this gene expression method to detect microbial activity in the cheese matrix was also demonstrated. Among the limitations of the methods, technological bias such as poor extraction or retrotranscription efficiencies could lead to the underestimation of cDNA copy numbers (Rantsiou et al. 2008a). This is a valuable

example of how a reverse transcription PCR-based method offers the ability to monitor microbial activities during cheese making, to understand how environmental stresses affect such activities, and to study the catabolism of substrates found in cheese by ripening cultures such as yeasts (Mansour et al. 2008). By RT-qPCR of RNA directly isolated from the cheese matrix, it is possible to increase our knowledge on gene expression leading to phenotypic features, especially during food fermentation and manufacturing. In this way, not only the presence of an organism but also some specific metabolic activities of interest could be measured, on the global microbial community, independently of cultivability. As an example, staphylococcal enterotoxin gene expression was rapidly detected in cheese (Duquenne et al. 2010). By a robust total RNA extraction method directly from the cheese, the authors have been able to quantify the RNA proportion of staphylococci and their transcript levels by RT-qPCR during the first 72 h of the cheese-making process. More recently, the expression of 11 nisin genes from *L. lactis* M78 in model cheeses was followed by RT-qPCR (Trmcic et al. 2011). Genes could be placed into four groups according to their expression over the time course of 24-h incubation of model UF (ultrafiltered milk) cheeses, reflecting the differential regulation of the transport and immunity genes (*nis*FEG) compared with the gene encoding nisin itself (*nis*A), its posttranslational modification and transport (*nis*BTCIP), or the regulatory genes *nisRK*. This study indicates that the quantification of the evolution of gene transcripts will be useful in the future to better understand the physiology of microorganisms growing in cheese. RT-qPCR analysis could be used to investigate possible interactions between different cheese-ripening microorganisms such as bacteria and yeasts (Mansour et al. 2009).

2.7 Transcription profiling

With the availability of whole-genome sequences, DNA array analysis offers the potential to monitor and compare the expression patterns of a wide range of mRNA species simultaneously (Smeianov et al. 2007; Xie et al. 2004). However, DNA macro- or microarray analysis is generally used to examine differential expression patterns of genes of a single microorganism resulting, for example, from changes in the microbial environment, e.g., conditions associated with dairy fermentations or cheese ripening (Cholet et al. 2008; Makhzami et al. 2008). DNA macroarrays were used to distinguish higher gene expression by a food isolate than a clinical isolate of *Enterococcus faecalis* in a model semi-hard cheese, indicating that the food isolate could be better adapted to the dairy environment (Makhzami et al. 2008). Notably, a targeted DNA microarray based on the published *L. lactis* subsp. *cremoris* SK11 genome sequence has been applied to four closely related strains of *L. lactis* subsp. *cremoris* during milk fermentation simulating the conditions of Cheddar cheese manufacture (Taïbi et al. 2010, 2011). With this approach, comparing transcriptomes provides a core expression profile that contributes to understanding gene expression responses to environmental variations, and the strain-specific responses allow the identification of markers for distinguishing among starter strains (Cretenet et al. 2011; Taïbi et al. 2011). Using a whole-genome oligonucleotide microarray for *L. lactis* subsp. *lactis* IL4103, Cretenet et al. (2011) have shown that the expression of autolytic prophage genes by *L. lactis* strain LD61 is delayed in UF cheese compared with

traditional cheese, which shows stronger autolysis. This may be due to the absence of severe stress conditions in the UF cheese model. Gene expression profiles also reflected responses to acid and oxidative stresses, as well as to the drop in temperature and carbon limitation, but not nitrogen starvation, due to the efficient proteolytic system. Multispecies microarrays have been used to identify major genes expressed by *Debaryomyces hansenii*, *Kluyveromyces marxianus*, and *Y. lipolytica* during growth in co-culture on methionine, lactate, and lactose in a cheese-like medium (Cholet et al. 2007). This study has shown that a microarray approach can be developed to investigate metabolic interactions between species within complex microbial ecosystem as well as to study the gene expression profile of a yeast in the presence of a specific substrate (Cholet et al. 2008).

2.8 Differential display techniques

Identification of differentially expressed genes can lead to greater insights into the molecular mechanism underlying diseases or other biological processes. The availability of the genome sequences of many microorganisms facilitates such research. The technologies derived from genomics such as DNA arrays are still rather expensive, beyond the reach of many research laboratories and require prior knowledge of the sequence information of the microorganisms of interest. In contrast, suppression subtractive hybridization (SSH) is a differential technique that does not require any sequence information of the particular strains. The SSH method has been applied to discriminating two *L. lactis* subsp. *cremoris* strains (SK11 and ATCC 19257), providing strain-specific gene markers that were useful for evaluating the relative ratios of these strains during mixed culture in milk and in complex M17 medium (Ndoye et al. 2011). During Cheddar cheese manufacture, various strains of *L. lactis* subsp. *cremoris* with specific characteristics are used as starters. To select the best combinations of strains according to their specific characteristics, it is important to identify signature sequences as well as to classify and distinguish strains. This approach will predict the metabolic performance of strains in co-culture during cheese manufacture by quantifying the expression of genetic markers. Although the ATCC 19257 genome is unknown, SSH permitted the isolation of 40% of specific genes as compared with those of SK11, whereas more than 50% of specific genes were found in the SK11 SSH library. Hence, a glycosyltransferase and amidase/aminoacylase (*amd*) genes were selected from the ATCC 19257 SSH library, whereas the gene LACR 1419 encoding a hypothetical protein was chosen from the SK11 SSH library for gene expression quantification by RT-qPCR. This made it possible to evaluate the RNA proportion of each strain in mixed culture by using the specific genes as biomarkers. Then, the transcriptional contribution of each strain was determined during co-culture in milk, thus giving the indication of their stability and activity. Such tools are useful for the dairy sector to improve the knowledge of starter strain diversity and performance during cheese fermentation or ripening.

In the absence of an available genome sequence, transcription profiling can be carried out by arbitrary-primed PCR, much in the same manner as RAPD profiles can be used to type strains. Fluorescent RNA arbitrarily primed PCR (FRAP-PCR) is a differential display method that has been applied to profile the transcriptome of *L. lactis* subsp.

cremoris strains during mixed culture in milk. Milk treatment by carbon dioxide influenced the transcription profiles of the defined culture, while neutralization by agitation was the best procedure compared with NaOH or Na_2CO_3 for regaining the original transcription profile at neutral pH (Dachet et al. 2010, 2011). In addition, lowering the rennet activity or increasing NaCl concentration greatly affected the transcription profile of the defined mixed culture. In combination with Förster resonance energy transfer PCR for discriminating individual strains, instability in strain ratios was demonstrated that could be related to the incompatibility of the different proteinase types of the strains (Dachet et al. 2011). Thus, FRAP-PCR could be used to screen strain compatibility and mixed culture associations of lactococcal strains, which would be difficult to accomplish by culture-dependent methods.

2.9 Proteomics and metabolomics

Proteomics in various forms has long been applied to dairy systems. In the review of Manso et al. (2005), the use of proteomic techniques was described for the characterization of milk products as well as proteins expressed by LAB. These types of approaches have been useful for discriminating the milk animal source and the genetic variants of milk proteins in Teleme cheese (Pappa et al. 2008). With pre-fractionation by RP-HPLC accompanied by MS and Edman degradation, peptides can be identified in each fraction, but major whey proteins can still mask minor components, as with 2D SDS-PAGE. Both major and minor components in water-soluble extracts were identified by combining N-terminal sequencing and LC/MS with quadruple time-of-flight MS, giving more complete profiles. Casein degradation can be monitored by identifying the peptides present in the cheese extract, thus indirectly portraying the proteolytic activity of the microbial community (Manso et al. 2005). Pre-fractionation steps have improved the resolution of proteomics in order to identify enzymes released into the cheese. Targeting the proteins produced by bacteria represents an additional step toward understanding the contribution of enzymatic activities to cheese manufacture and ripening. As reviewed by Gagnaire et al. (2009), although proteomics has been applied to milk and cheese over the past decade, the last few years have seen the challenge of quantitative proteomics beginning to produce results with cheese. Gagnaire et al. (2004) showed the release of stress proteins originating from *P. freudenreichii*, *Lactobacillus helveticus*, and *S. thermophilus* during the ripening of Emmental cheese. Yvon et al. (2011) were the first to combine both metabolomics and proteomics to compare two *L. lactis* strains in the solid matrix of UF model cheese. Differences between strains were observed in the activity and induction of the proteolytic system. Low rates of casein degradation and peptide transport lead to amino acid limitation in the case of strain LD61. Common responses to the cheese environment over 7 days of ripening were thus related to acidification and amino acid starvation.

Metabolomics carried out by Fourier transform infrared spectroscopy succeeded in discriminating among strains of *D. hansenii* with identical RAPD profiles and when 26S rRNA sequencing showed no strain differences (Del Bove et al. 2009). However, metabolomic profiles could not be grouped according to geographical locale where the Pecorino cheese was produced.

3 Culture-independent methods: future directions

Next-generation DNA sequencing such as pyrosequencing allows high throughput, is less labor-expensive, and should be a powerful culture-independent method for food samples in the future (Shendure and Ji 2008). This approach eliminates the need for cloning and culture-dependent methods, thereby avoiding both production of aberrant recombinants and cloning-related artifacts (Speksnijder et al. 2001). The pyrosequencing technique has been applied for the first time in food ecosystems by Humblot and Guyot (2009) and thus shows promise for culture-independent analysis that could be applied to the cheese microbial ecosystem. These authors have investigated the feasibility of analyzing microbiomes of fermented foods using different pearl millet slurries mixed with groundnuts. A highly variable region of the 16S rRNA gene is amplified from the total DNA or cDNA extracted from slurries using primers that target adjacent conserved regions, followed by direct sequencing of individual PCR products. From this study, the pyrosequencing technique enabled the characterization of 137,469 sequences of 16S rRNA gene amplicons of cultivable bacteria in which Firmicutes such as LAB were mainly represented. More recently, both DNA and cDNA extracted from Danish raw milk cheeses were analyzed by 16S rRNA pyrosequencing, showing that low-frequency community members could be detected more consistently by pyrosequencing than by DGGE (Masoud et al. 2011). Pyrosequencing gave a reliable estimate of the relative abundance of bacterial species, although the percentages of the main bacteria from the total sequences were different between 16S rDNA and cDNA libraries of the same samples. In contrast, Humblot and Guyot (2009) found that the numbers of sequences obtained from the 16S cDNA libraries were lower than those obtained from the 16S rDNA libraries. This difference could be explained by a bias in PCR amplification or to the imprecise quantification of the PCR products leading to strong deviation during pooling of samples (Humblot and Guyot 2009). The pyrosequencing approach offers a more global view of the community structure including metabolically active microorganisms. Sequencing of food genomes and transcriptomes provides deeper insight into microbial diversity and might allow a better understanding of the metabolic and biochemical potential of the studied communities.

4 Conclusion

Culture-independent methods have arisen to face the multiple limitations of traditional cultivation in the analysis of microbial community structure, diversity, and activity. Common molecular techniques are being used to analyze the genetic material from microorganisms in food samples. Nowadays, through culture-independent methods, challenging foods such as cheeses are more easily studied to understand their complex ecosystem. Literature on culture-independent methods has only begun to show the composition of the cultivable microbiota in cheeses, and their potential should discover novel pathways for uncultivable microorganisms in the cheese matrix. The target mRNA genes as potential probes to study in situ gene expression of cheese matrix are of great interest to learn more about the uncultivable

microorganisms and why certain microorganisms are uncultivable and to transform them to cultivable systems. For this purpose, improved bioinformatics will be required for sequence profiling. However, due to the biases of molecular methods, culture-dependent and culture-independent approaches should be combined as much as possible through polyphasic characterization to undertake the analysis of both the community and activity of total resident lactic acid bacteria as well as spoilage agents in cheese or dairy products. In the near future, next-generation DNA sequencing will enable to add a molecular dimension to understanding flavor, taste, and texture of cheese during ripening.

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