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Review

Molecular approaches to analysing the microbial composition of raw milk and raw milk cheese

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ABSTRACT

The availability and application of culture-independent tools that enable a detailed investigation of the microbiota and microbial biodiversity of food systems has had a major impact on food microbiology. This review focuses on the application of DNA-based technologies, such as denaturing gradient gel electrophoresis (DGGE), temporal temperature gradient gel electrophoresis (TTGE), single stranded conformation polymorphisms (SSCP), the polymerase chain reaction (PCR) and others, to investigate the diversity, dynamics and identity of microbes in dairy products from raw milk. Here, we will highlight the benefits associated with culture-independent methods which include enhanced sensitivity, rapidity and the detection of microorganisms not previously associated with such products.

Contents

1. Introduction

Raw milk is known to harbour a complex microbial community. Indeed the high nutritional value of this food, its high water content and near neutral pH allows the growth of many microbes (Frank, 1997). These microorganisms include bacteria of technological relevance such as the lactic acid bacteria (LAB), which can contribute to subsequent desirable fermentative reactions (Fox, 1999). However the presence of spoilage bacteria can have considerable negative effects on the quality of milk and dairy products (Cousin, 1982) while the presence of pathogens can have more severe repercussions. The traditional means of determining the nature of the microbiota present in milk is culture based. The culturable microbiota of milk consists primarily of LAB such as *Lactobacillus*, *Streptococcus*, *Enterococcus*, *Lactococcus*, *Leuconostoc*, *Weisella* and

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Table 1
Description of the main genomic-based methods involved in describing microorganisms in milk and cheese. Detailed descriptions of these principals have been reviewed recently by Juste et al. (2008), Pogacic et al. (2010) and Randazzo et al. (2009a, 2009b).

Method	Principal
Culture dependent genotyping methods	
Random Amplified Polymorphic DNA—RAPD	Uses short arbitrary primers and low-stringency hybridisation to randomly amplify DNA fragments which are separated to give a fingerprint pattern
Restriction Fragment Length Polymorphisms—RFLP	A profiling tool based on digestion of amplified ribosomal DNA using one or more restriction enzymes. Another name for this method is, ARDRA—Amplified Ribosomal DNA Restriction Analysis.
Culture independent molecular methods	
Denaturing or Temporal Temperature Gradient Gel Electrophoresis—DGGE or TTGE	The separation of small PCR amplicons, distinguished by differences in their DNA sequences. Amplicons are separated from a low to high gradient, in the direction of the electrophoresis. DGGE uses a chemical gradient (urea or formamide). TTGE has a temperature gradient and a constant concentration of denaturants
Single Stranded Conformation Polymorphisms—SSCP	Allows separation of different DNA fragments of similar length on the basis of conformational differences in folded single stranded products and visualised on gels or as peaks using an automated sequencer
Real-Time PCR—qPCR	Uses a fluorescent probe to monitor amplification of the target DNA in real-time and enables quantification of a target species. Uses species-specific primers to target a gene/organism
Intergenic Transcribed Spacer Analysis—ITS	Analyses the bacterial ITS region located between the 16S and 23S ribosomal genes allowing differentiation between strains of the same species or closely related species.
Automated Ribosomal Intergenic Spacer Analysis—ARISA	A similar method to ITS but uses a fluorescent primer in the amplification of microbial ribosomal intergenic spacers. It generates peaks which correspond to discrete DNA fragments detected by a fluorescence detection system
Terminal Restriction Fragment Length Polymorphisms—T-RFLP	Based on the digestion of fluorescent, end-labelled, PCR products with restriction endonucleases after electrophoretic separation, the end-labelled terminal restriction fragments are compared with DNA size standards. Variation in the presence and location of the restriction sites result in different groups having different fragment lengths
Fluorescence In Situ Hybridisation—FISH	Bacterial cells hybridise to a fluorescently labelled DNA probe and can be detected and counted by fluorescence microscopy techniques
Denaturing High Performance Liquid Chromatography—DHPLC	Separates PCR amplicons using an ion-pair revered-phase high performance liquid chromatography automated detection system.
Length Heterogeneity PCR—LH PCR	Employs a fluorescently labelled oligonucleotide as the forward primer, coupled with an unlabelled reverse pair to amplify hyper-variable regions. Labelled fragments are separated and detected by fluorescence with an automated sequencer.

Pediococcus. While strains of other genera such as *Propionibacterium*, *Staphylococcus*, *Corynebacterium*, *Brevibacterium* also occur, yeasts and moulds may also be present (Coppola et al., 2008), there also exists a large spectrum of other microbes which occur less frequently or are more difficult to detect. Culture-based methods rely on the isolation and cultivation of microorganisms prior to their identification on the basis of phenotype or genotype. However, it has become apparent that approaches that include a culturing step can lead to inaccuracies due to species present in low numbers being out-competed in laboratory media by numerically more abundant microbial species (Hugenholtz et al., 1998) or the fact that others may simply not be amenable to cultivation in the laboratory (Head et al., 1998). For these reasons approaches to assess the microbial composition of food have had to change dramatically. To address this, there has been an increased focus in recent years on the use of culture-independent investigations through the direct analysis of DNA (or RNA) from food without a culturing step (Table 1; Fig. 1) (Coppola et al., 2008; Jany and Barbier, 2008). These represent rapid, sound, reliable and effective methods for the detection and identification of the microorganisms present in dairy products.

2. Culture-independent analysis

Culture-independent approaches have been used increasingly to determine the composition of complex microbial communities. These procedures have enabled the simultaneous characterisation of whole ecosystems and the identification of many species from these sources. The shift from culture-dependent assessment to culture-independent analysis has led to a revolution in microbial ecology. These techniques provide a more sensitive and rapid method than conventional culture-

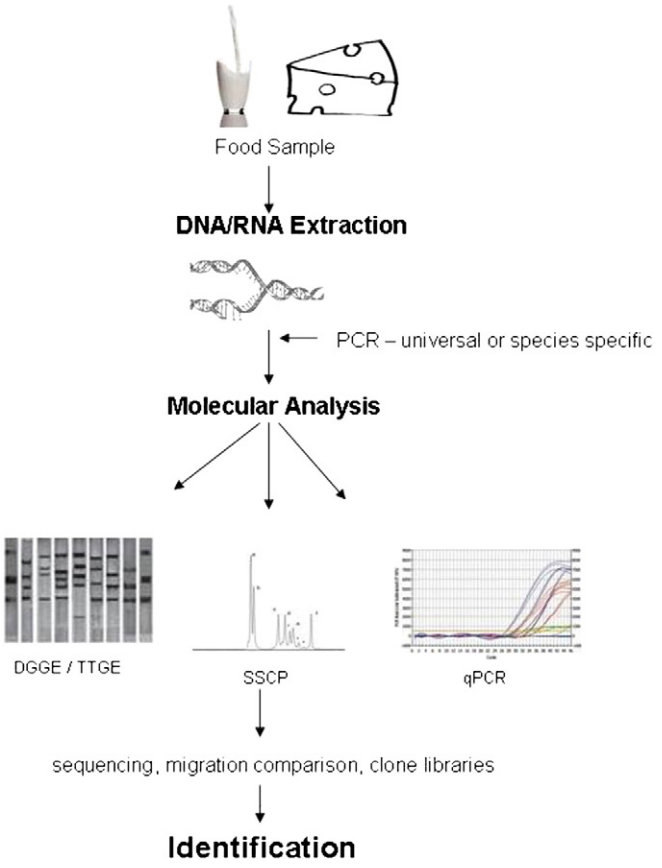

Fig. 1. Overview of the steps involved in a culture-independent assessment of a food system. This figure highlights the major points to consider for some the main culture-independent methods. Other methods, such as FISH or DHPLC, may involve different processes.

dependent analysis with the major benefit of detecting microorganisms which are difficult to culture or uncultivable. Unfortunately, while such approaches possess numerous advantages over culture-based methods, there can still be some limitations. A number of key factors to consider when employing a culture-independent approach are described below.

2.1. Differentiating between the DNA of live and dead microorganisms

Culture-independent assessment most frequently relies on the analysis of nucleic acids isolated from an entire microbial population. DNA is the focus of analysis in the majority of such studies and provides information with respect to the bacterial diversity and overall microbial history of the environment in question. However, the analysis of DNA does not typically enable one to distinguish that from living and dead cells. In recent years there has been an increased use of DNA stains, such as ethidium monoazide (EMA) (Rudi et al., 2005) or propidium monoazide (PMA) (Josefsen et al., 2010), which penetrate and stain the DNA of dead cells and prevent the subsequent PCR amplification thereof. Alternatively, RNA can be used as a live-cell specific target which also allows one to monitor the active microflora (Alessandria et al., 2010; Duthoit et al., 2005; Rantsiou et al., 2008b). While RNA-based studies frequently provide the same results as their DNA-based counterparts, they can on occasion facilitate the identification of microbes not detected by DNA (Alessandria et al., 2010). Thus RNA-based analysis can provide a greater understanding of microbial community structure and functionality (Bodrossy et al., 2006).

2.2. Nucleic acid extraction

In the case of culture-independent approaches, the outcome is dependent on the extraction of DNA (or RNA) which is representative of the total microbial population and is of sufficiently high concentration and purity. This can be problematic as the presence of natural compounds such as fats, carbohydrates, proteins and salts can hamper nucleic acid extraction as well as downstream application (Wilson, 1997). However, nucleic acid extraction can be improved by the inclusion of various steps such as the mechanical or enzymatic lysis of cells (Lafarge et al., 2004; Parayre et al., 2007), protein digestion (Parayre et al., 2007) and DNA precipitation (Duthoit et al., 2003). More recently the application of commercially available DNA extraction kits has yielded highly pure DNA (Kuang et al., 2009) while reducing laboratory time and removing the need for harmful chemicals.

2.3. Target region

Following the extraction of nucleic acids from the food matrix, the majority of investigations to date have relied on the use of PCR to amplify the region of interest (Fig. 1). The most commonly employed targets for identifying species are the 16S and 26S ribosomal RNA (rRNA)-encoding genes, or regions thereof, for bacterial and eukaryote identification, respectively (Cocolin et al., 2002; Florez and Mayo, 2006). These are specifically targeted as a consequence of possessing both highly conserved and highly variable domains. The existence of conserved regions facilitates the use of universal PCR primers to amplify portions of the gene while analysis of the hypervariable regions allows the identification of the corresponding microorganisms (Delbes and Montel, 2005). In situations where only a region of the 16S rRNA gene is targeted, the V3 region is most commonly amplified (Callon et al., 2007; Delcenserie et al., 2007; Ogier et al., 2002; Randazzo et al., 2010). However, some authors have suggested that other regions can provide a more in-depth assessment (Aponte et al., 2008; Randazzo et al., 2006). Alternatively, one can target other genes such as those encoding the phenylalanine tRNA synthase (*pheS*) (Zago et al., 2009) or the RNA polymerase B subunit (*rpoB*) (Martin-Platero et al., 2009). It should also be noted that while biases, such as the introduction of heteroduplexes (Kanagawa, 2003) or chimeric amplicons (Wang and Wang, 1997), may occur during subsequent PCR reactions, these can be minimised through the use of high-quality primers, high-fidelity polymerases and by modifying PCR conditions (Ogier et al., 2002).

2.4. Choice of culture-independent technique

A variety of different methods, such as DNA sequencing, denaturing gradient and/or temporal temperature gradient gel electrophoresis (DGGE/TTGE) or single stranded conformation polymorphisms (SSCP) are employed to differentiate between 16S/26S amplicons with different signature sequences. In-depth details of the principal for each of these techniques have been reviewed recently (Coppola et al., 2008; Jany and Barbier, 2008; Juste et al., 2008; Pogacic et al., 2010; Randazzo et al., 2009a; Trmcic et al., 2008) and a summary of these can be found in Table 1. When choosing a technique, the question being asked must be considered. These technologies may be employed to study the general microbial diversity of an ecosystem (Bonetta et al., 2008; Callon et al., 2007; Duthoit et al., 2003), to identify specific microorganisms present (Delbes and Montel, 2005) or both (Martin-Platero et al., 2009). They may also be used to assess microbes in a semi-quantitative, e.g. DGGE or T-RFLP (Terminal Restriction Fragment Length Polymorphism) (Randazzo et al., 2010; Sanchez et al., 2006), or quantitative, e.g. qPCR (quantitative real-time PCR) (Rasolofo et al., 2010), manner. Alternatively, where an assessment of the distribution of microorganisms in a food matrix is required, techniques such as fluorescent in-situ hybridisation (FISH) may be utilised (Ercolini et al., 2003).

Although quantitative methods have many benefits, which, in addition to the ability to quantify, include enhanced precision and specificity, there can be disadvantages. qPCR cannot effectively simultaneously quantify very large numbers of different targets in a single sample and therefore selection of target genes and the development of specific primers and probes is vital (Juste et al., 2008). Furthermore, in many instances poor detection and a lack of reproducibility can be problems in situations where cell numbers are low and it is thus critical that stringent detection methods are designed (Rantsiou et al., 2008a).

It should be noted that approaches that rely on electrophoretic patterns can, on occasion, suffer from resolution-related issues. These problems can be reduced by, for example, the addition of a GC-clamp to one of the primers to increase resolution when using DGGE (Sheffield et al., 1989). However, problems can persist if the melting behaviour of 16S fragments are highly similar/identical while there can also be a concern that multiple band display due to multiple rRNA copy numbers will result in diversity being overestimated (Ercolini, 2004). These problems may be overcome through careful primer design and the application of different sets of primers has also been shown to reduce these occurrences (Duthoit et al., 2003). A benefit of the use electrophoretic methods has been that it has provided the possibility to subsequently excise bands from gels, facilitating DNA sequencing and the identification of microbes through comparisons with specific reference strains (Parayre et al., 2007), or, more routinely, using public databases such as GenBank or the Ribosomal Database Project (RDP) (Giannino et al., 2009; Ogier et al., 2004). To date, the use of high-throughput DNA sequencing to directly sequence multiple 16S amplicons simultaneously has not been extensively employed to investigate the diversity of milk and cheese.

3. Application of culture-independent methods to study the microbiota and diversity of milk and cheese

3.1. Evaluation of the microbial diversity of milk

One of the most detailed culture-independent studies of a dairy related food was by Callon et al. (2007), who examined the microbial diversity of goats milk samples throughout one lactation year. This study

Table 2
Comparison of studies employing both culture-dependent and culture-independent techniques for the analysis of microbial communities of raw milk and cheese.

Authors	Method	Culture dependent microorganisms	Culture independent microorganisms	Substrate
Randazzo et al., 2002	DGGE	*Lactococcus lactis* *Leuconostoc mesenteroides*[a] *Lactobacillus fermentum/plantarum/casei*[a] *Pediococcus acidilactici*[a] *Enterococcus sulfurans/faecalis* *Streptococcus thermophilus*[a] *Enterococcus hirae*[a]	*Lactococcus lactis* *Leuconostoc mesenteroides* *Lactobacillus fermentum/plantarum/casei/delbrueckii* subsp. *bulgaricus* *Pediococcus acidilactici* *Enterococcus faecalis/hirae/sulfurans* *Streptococcus thermophilus/bovis* *Macrococcus caseolyticus*	Cows milk cheese
Cocolin et al., 2002	DGGE	*Candida catenulata/pararugosa/parapsilosis/zeylanoides/pseudointermedia/rugosa* *Cryptococcus curvatus* *Kluyveromyces marxianus/lactis* *Pichia guilliermondii* *Saccharomyces cerevisiae* *Trichosporon mucoides*	*Candida pseudorugosa/kefyr/pseudointermedia/humulis/rugosa* *Galactomyces spp.* *Kluyveromyces marxianus/lactis* *Saccharomyces bayanus/cerevisiae*	Cows milk
Henri-Dubernet et al., 2004	TTGE	*Lactobacillus paracasei* subsp. *paracasei/delbrueckii* subsp. *bulgaricus/delbrueckii* subsp. *lactis/casei* subsp. *casei/acidophilus/plantarum*[b]	*Lactobacillus paracasei* subsp. *paracasei/plantarum*	Cows milk cheese
Feurer et al., 2004	SSCP	*Arthrobacter arilaitensis* *Streptococcus thermophilus* *Lactococcus lactis* subsp. *lactis* *Brevibacterium linens* *Carnobacterium maltaromaticum* *Marine bacterium* *Corynebacterium casei* *Lactobacillus curvatus* subsp. *curvatus* *Marinolactibacillus psychrotolerans* *Microbacterium gubbeenense* *Brachybacterium species*	*Arthrobacter arilaitensis* *Streptococcus thermophilus* *Lactococcus lactis* subsp. *lactis/lactis* subsp. *cremoris* *Brevibacterium linens* *Carnobacterium maltaromaticum* *Marine bacterium* *Corynebacterium casei* *Lactobacillus curvatus* subsp. *curvatus, sakei* *Marinolactibacillus psychrotolerans* *Microbacterium gubbeenense* *Brachybacterium species* *Pseudoalteromonas species* Uncultured Flavobacterium	Cows milk cheese
Callon et al., 2006	SSCP	*Kluyveromyces lactis/marxianus* *Kluyveromyces* *Candida zeylanoides/parapsilosis/silvae/intermedia/tropicalis/rugosa* *Debaryomyces hansenii* *Saccharomyces cerevisiae/unisporus* *Pichia guilliermondii*	*Kluyveromyces lactis/marxianus* *Kluyveromyces* *Candida zeylanoides/parapsilosis/silvae/intermedia* *Debaryomyces hansenii* *Saccharomyces cerevisiae/unisporus*	Cows milk cheese
Delbes et al., 2007	SSCP	*Streptomyces species* *Brachybacterium species* *Enterococcus faecalis*[c] *Bacillus pumilus* *Staphylococcus pasteuri/haemolyticus* *Lactobacillus casei* *Streptococcus dysgalactiae/thermophilus*[c] *Lactococcus lactis*[c] *Lactococcus garvieae* *Microbacterium oxydans/lacticum/laevaniformans* *Sphingomonas species* *Chryseobacterium* species[c] *Flavobacterium species*[c] *Luteibactor rhizovicinus*[c] *Psychrobacter species* *Moraxella osloensis* *Stenotrophomonas maltophilia*[c] *Brevibacterium linens*[c] *Kocuria rhizophila/carniphila*[c] *Arthrobacter arilaitensis*[c] *Corynebacterium flavescens*[c] *Staphylococcus fleurettii/saprophyticus/vitulinus/epidermidis/equorum/pasteuri*[c] *Aerococcus viridans*[c] *Marinilactibacillus psychrotolerans*[c] *Brevundimonas nasdae* *Enterobacter agglomerans*[c] *Klebsiella oxytoca/terrigena/trevisani*[c] *Luteibactor rhizovicinus*[c] *Psychrobacter faecalis* *Enterobacter aerogenes* *Citrobacter freundii*	*Actinobacteria* *Kocuria rhizophila* *Corynebacterium species* *Microbacterium foliorum* *Dietzia maris* *Dietzia sp.* *Nocardioides dubius* *Corynebacterium confusum* *Arthrobacter psychrolactophilus* *Leucobacter komagatae* *Corynebacterium xerosis* *Clostridium glycolicum/lituseburense* *Enterococcus faecalis* *Staphylococcus warneri/equorum* *Turicibacter sanguinis* *Jeotgalicoccus psychrophilus* *Lactobacillus casei/kefiranofaciens* *Streptococcus dysgalactiae/thermophilus* *Facklamia tabacinasalis* *Lactococcus lactis* *Mesorhizobium amorphae* *Bradyrhizobium japonicum* *Enterobacter agglomerans* *Ralstonia pickettii* *Alcaligenes sp.* *Acinetobacter lwoffii* *Chryseobacterium* sp. *Sphingobacterium sp.*	Cows milk cheese

Table 2 (*continued*)

Authors	Method	Culture dependent microorganisms	Culture independent microorganisms	Substrate
		Moraxella osloensis[c] *Streptococcus parauberis*		
Callon et al., 2007	SSCP	*Staphylococcus epidermidis/simulans/caprae/equorum* *Kocuria rhizophila/Kristinae carniphila* *Bacillus thuringiensis-cereus* *Micrococcus species* *Brevibacterium stationis* *Microbacterium oxydans* *Exiguobacterium* *Corynebacterium variable* *Brachybacterium paraconglomeratum* *Arthrobacter sp.* *Salinicoccus sp.* *Jeogalicoccus psychrophiles* *Micrococcus caseolyticus* *Ornithinicoccus sp.* *Dietza maris* *Rothia sp.* *Clostridium* *Enterococcus faecalis* *Lactococcus lactis/garvieae* *Lactobacillus casei* *Leuconostoc mesenteroides* *Streptococcus mitis* *Enterococcus saccharominimus* *Pantoea agglomerans* *Pseudomonas putida/aeruginosa/fulgida* *Acinetobacter baumanii* *Citrobacter freundii* *Stenotrophomonas maltophilia* *Chryseobacterium indologenes* *Delftia acidovorans* *Enterobacter sp./absuria* *Hahella chejuensis* *Klebsiella milletis-oxytoca* *Pseudomonas* *Candida* *Cryptococcus* *Debaryomyces* *Kluyveromyces* *Rhodotorula* *Trichosporon*	*Staphylococcus epidermidis/caprae/simulans/equorum/* *Kocuria rhizophila/Kristinae carniphila* *Bacillus thuringiensis-cereus* *Micrococcus sp.* *Brevibacterium stationis* *Microbacterium oxydans* *Exiguobacterium* *Corynebacterium variable* *Brevibacterium stationis* *Brachybacterium paraconglomeratum* *Arthrobacter sp.* *Salinicoccus sp.* *Jeogalicoccus psychrophiles* *Micrococcus caseolyticus* *Ornithinicoccus sp.* *Dietza maris* *Rothia sp.* *Enterococcus faecalis/saccharominimus* *Lactococcus lactis/garvieae* *Lactobacillus casei* *Leuconostoc mesenteroides* *Streptococcus mitis* *Pantoea agglomerans* *Pseudomonas putida/aeruginosa* *Acinetobacter baumanii* *Citrobacter freundii* *Stenotrophomonas maltophilia* *Chryseobacterium indologenes* *Delftia acidovorans* *Enterobacter sp./absuria* *Hahella chejuinsis* *Klebsiella milletis-oxytoca* *Candida* *Cryptococcus* *Debaryomyces* *Kluyveromyces* *Rhodotorula* *Trichosporon*	Goats milk
Van Hoorde et al., 2008	DGGE	*Lactococcus lactis* subsp. *lactis*[a] *Lactobacillus paracasei/plantarum/brevis/curvatus*[a] *Pediococcus pentosaceus*[a] *Lactobacillus rhamnosus/perolens* *Streptococcus salivarius* *Weissella paramesenteroides*	*Lactococcus lactis* subsp. *lactis* *Lactobacillus paracasei/plantarum/brevis/curvatus/rhamnosus/parabuchneri/gallinarum* *Pediococcus pentosaceus* *Enterococcus faecalis*	Cows milk cheese
Nikolic et al., 2008	DGGE	*Lactobacillus paracasei* subsp. *paracasei* *Lactococcus lactis* subsp. *lactis* *Enterococcus faecalis*	*Lactobacillus paracasei* subsp. *paracasei* *Lactococcus lactis* subsp. *lactis* *Enterococcus faecalis* *Leuconostoc mesenteroides*	Goats milk cheese
Gala et al., 2008	DGGE	*Lactobacillus casei/buchneri*[a] *Lactobacillus paracasei* subsp. *paracasei/tolerans/rhamnosus* *Pediococcus acidilactici*[a]	*Lactobacillus casei/delbrueckii* subsp. *lactis/parabuchneri/fermentum/rhamnosus*	Cows milk cheese
Dolci et al., 2008	DGGE	*Lactococcus lactis* subsp. *lactis*/subsp. *cremoris* *Lactococcus lactis* *Lactobacillus plantarum/paracasei/casei/coryneformis* subsp. *torquens/delbrueckii* subsp. *lactis* *Enterococcus faecium* *Enterococcus faecalis*	*Lactococcus lactis* subsp. *lactis*/subsp. *cremoris* *Lactococcus lactis* *Lactobacillus plantarum/kefiranofaciens* *Macrococcus caseolyticus* *Streptococcus agalactiae*	Cows milk cheese
Henri-Dubernet et al., 2008	TTGE	*Lactobacillus paracasei/helveticus/rhamnosus/parabuchneri/fermentum/perolens/acidophilus/brevis/kefiri/delbrueckii* subsp. *bulgaricus/delbrueckii* subsp. *lactis*[b]	*Lactobacillus paracasei/plantarum/acidophilus/rhamnosus*	Cows milk cheese

(*continued on next page*)

Table 2 (*continued*)

Authors	Method	Culture dependent microorganisms	Culture independent microorganisms	Substrate
Aponte et al., 2008	DGGE	*Lactococcus lactis*[a] *Streptococcus thermophilus*[a] *Enterococcus faecalis/durans/faecium*[a] *Streptococcus parauberis* *Streptococcus haemolyticus/croceolyticus/warneri/pasteuri*[a] *Shigella boydii*[a] *Staphylococcus aureus*[a] *Macrococcus caseolyticus*[a] *Lactobacillus helveticus/delbrueckii* subsp. *lactis/delbrueckii* subsp. *bulgaricus/delbrueckii* subsp. *delbrueckii/delbrueckii* subsp. *indicus/fermentum*[a] *Lactobacillus paracasei/rhamnosus* *Lactococcus garvieae* *Leuconostoc mesenteroides* subsp. *lactis*[a] *Pediococcus acidilactici*[a]	*Lactococcus lactis* *Streptococcus thermophilus* *Macrococcus caseolyticus* *Morexella osloensis* *Weisella species* *Lactobacillus helveticus/delbrueckii* subsp. *lactis* *Rahnella species* *Aeromonas simiae* *Staphylococcus aureus/haemolyticus*	Cows milk cheese
Abriouel et al., 2008	TTGE	*Lactobacillus paracasei/plantarum/brevis* *Lactococcus lactis* *Leuconostoc mesenteroides/pseudomesenteroides* *Enterococcus devriesei/faecium* *Enterococcus* *Pediococcus urinaequi* *Hafnia alvei* *Escherichia coli* *Obesumbacterium proteus* *Shigella flexneri*	*Lactobacillus plantarum/brevis/acidophilus/paracasei* *Enterococcus species* *Lactococcus lactis* *Escherichia coli* *Nitrogen-fixing bacterium*	Goat and sheep milk cheese
Rantsiou et al., 2008a, 2008b	DGGE	*Lactobacillus planatrum/brevis/coryneformis/paraplantarum* *Kluyveromyces lactis* *Pichia membranifaciens/fermentans* *Candida krisii/zeylanoides*	*Streptococcus thermophilus* *Lactobacillus delbrueckii* subsp. *bulgaricus /plantarum/helveticus/suntoryeus/gallinarum* *Lactococcus lactis* *Kluyveromyces lactis* *Pichia membranifaciens/fermentans* *Candida zeylanoides*	Sheep and goat milk cheese
Serhan et al., 2009	DGGE	*Lactobacillus curvatus/plantarum* *Lactobacillus* *Enterococcus faecium/durans/faecalis/malodoratus* *Lactococcus lactis* subsp. *lactis* *Streptococcus thermophilus*	*Lactobacillus plantarum* *Enterococcus faecium/durans/faecalis/malodoratus* *Lactococcus lactis* subsp. *lactis* *Staphylococcus haemolyticus* *Streptococcus species* *Escherichia coli* *Clostridium bifermetans/Eubacterium tenue* Unidentified bands	Goats milk cheese
Randazzo et al., 2009a, 2009b	DGGE	*Lactobacillus rhamnosus/brevis* *Leuconostoc mesenteroides* *Lactococcus lactis* *Streptococcus thermophilus* *Enterococcus faecalis*	*Lactobacillus rhamnosus/brevis/plantarum/pentosus/fermentum/buchneri/delbrueckii* *Leuconostoc mesenteroides* *Lactococcus lactis* *Streptococcus thermophilus* *Enterococcus faecalis*	Sheep's milk cheese
Alegria et al., 2009	DGGE	*Lactococcus lactis/garvieae* *Staphylococcus saprophyticus/pasteuri* *Klebsiella species* *Lactobacillus plantarum* *Escherichia coli* *Micrococcus luteus* *Corynebacterium variable* *Flavobacterium species* *Leuconostoc mesenteroides* *Microbacterium oxydans* *Musa acuminata*	*Streptococcus thermophilus* *Lactococcus lactis/garvieae* *Streptococcus parauberis/uberis/iniae* *Lactobacillus plantarum/casei/paracasei* *Enterococcus faecium* *Corynebacterium variable* *Macrococcus caseolyticus* *Geotrichum candidum* *Kluyveromyces sp.* *Saccharomyces sp.* *Trichosporon gracile*	Cows milk cheese
Alessandria et al., 2010	DGGE	*Lactococcus* subsp. *lactis/garvieae* *Enterococcus faecium/casseliflavus/faecalis/italicus/durans* *Lactobacillus pentosus/brevis/plantarum* *Staphylococcus capitis/parauberis/epidermis* *Macrococcus caseolyticus* *Leuconostoc citreum/mesenteroides* *Pediococcus pentosaceus* *Weissella paramesenteroides*	*Lactococcus lactis* subsp. *lactis* *Lactobacillus helveticus* *Leuconostoc pseudomesenteroides* *Delphinella strobiligena* *Escherichia coli* *Propionibacterium acnes* *Methylobacterium sp.* *Gluconobacter thailandicus* *Moraxella oslonesis*	Goats milk cheese

Table 2 (*continued*)

Authors	Method	Culture dependent microorganisms	Culture independent microorganisms	Substrate
		Candida paraugosa/zeylanoides/parapsilosis *Aerobasidium pullulans* *Cryptococcus sp.* *Discophareina fagi* *Rhodotorula glutinis* *Debaromyces hansenii* *Trichosporon coremiiforme*	*Kocuria rhizophila* *Klebsiella sp.* *Aureobasidium pullulans* *Phoma herbarum* *Seyrigia humbertii* *Saccharomyces cerevisiae* *Filobasidium/Cryptococcus sp.* *Candida pararugosa* *Rhizomucor miehei* *Alternaria alternata*	
Dolci et al., 2010	DGGE	*Lactococcus lactis* subsp. *lactis*/ subsp. *cremoris*[a] *Lactococcus lactis* *Lactobacillus casei/plantarum/coryneformis* subsp. *torquens/acidipiscis*[a] *Streptococcus agalactiae*[a] *Streptococcus thermophilus*[a]	*Lactococcus lactis* subsp. *lactis*/subsp. *cremoris* *Lactobacillus casei/helveticus* *Streptococcus agalactiae* *Spingomonas species*	Cows milk cheese

Subscripts a, b, c indicate that DGGE, TGGE or SSCP, respectively, were also used in identifying these isolates.

relied on SSCP analysis of DNA extracted directly from milk and from isolates, as well as RFLP (Restriction Fragment Length Polymorphisms) typing of isolates. The combined use of these techniques, as well as culturing on a wide selection of media, revealed the presence of a diverse population of bacteria and yeast in the milk (Table 2). In addition to species commonly encountered in milk, some species which are atypical of goats milk or had previously only been associated with cheeses, including a number of corynebacteria and brachybacteria, were identified thereby highlighting the sensitivity of this approach. Another unexpected finding was the detection of several halophilic species atypical of milk, including *Jeotgalicoccus psychrophilus*, *Salinicoccus* sp., *Dietzia maris*, *Exiguobacterium*, *Ornithinicoccus* sp. and *Hahella chejuensis*, these were detectable by RFLP and SSCP of isolates and from milk DNA extracts. While the two techniques employed each identified many of the same species, the culture based approach was considerably more labour intensive, involving the use of 9 different media, isolate selection, purification and storage. In contrast, SSCP facilitated the rapid detection of the same species (Callon et al., 2007). The analysis carried out also highlighted a seasonal variation in the microbial composition of the milk. This is an important factor when considering the ultimate use of the milk as, for example, some variations may affect the flavour development of cheese (Randazzo et al., 2010). Another factor which has been found to influence the microbial composition of milk is the location of the animal. More specifically, Bonizzi et al. (2009) employed intergenic transcribed spacer analysis (ITS; Table 1) to investigate the composition of milk sampled at different regions, i.e. alpine pasture, valley and lowland farms, of the North-western Italian Alps over a 2-year period. While this approach did not reveal the identity of the species or strains present, evident differences in band patterns between lowland plains, valleys and alpine pastures were apparent. When analysed yearly, cluster analysis indicated that the milk from alpine pastures formed a large, nearly homogenous cluster, while that from valleys showed a tendency to scatter into a number of small mixed groups with some adjoining the alpine pasture cluster. It was noted, however, that some bands were common across the samples suggesting that a number of species were widespread (Bonizzi et al., 2009). This technique was also employed to establish that farm location impacts on the microbial composition of cheese. This is an important consideration with respect to the discrimination of cheeses especially in the context of Protected Designation of Origin (PDO) cheese (Bonizzi et al., 2007). A more recent study has also highlighted the impact of a cows feeding environment on milk microbial composition. Here, DGGE and qPCR were employed to reveal that the predominant species, regardless of whether feeding occurred indoor and outdoor, were lactobacilli, but that *Staphylococcus* species appeared in milk after 8 days of outdoor feeding only (Hagi et al., 2010). Finally, it is also important to consider the yeast population of milk, which can impart important flavours on dairy products (De Freitas et al., 2009). Cocolin et al. (2002) carried out such analyses which focused on the yeast population in raw cows milk and used a combination of culturing and DGGE fingerprinting. The classical culture-based methods identified six *Candida* species (which accounted for 56.2% of the population), two *Kluyveromyces* species, *Saccharomyces cerevisiae*, *Pichia guilliermondii*, *Trichosporon mucoides* and *Cryptococcus curvatus* as being the main yeast constituents. When the samples were analysed by DGGE additional components such as *Galactomyces* sp., *Candida kefyr*, *Candida humilis* and *Saccharomyces bayanus/pastorianus* were detected (Table 2) (Cocolin et al., 2002).

3.2. Evaluation of the microbial diversity of whey starters

While the manufacture of cheese frequently can involve fermentation by the natural microbial population only, i.e. no starter culture addition, there are many cheeses which use starter cultures. These may be defined starters, a known culture or a mix of known cultures which contribute to specific cheese traits during manufacture or in some instances natural whey starters (NWS). NWS have a complex microbial association of various species as well as a large number of biotypes (Giraffa et al., 1997). The complex microbiota of these whey starters has been investigated in recent years using culture-independent approaches. In one instance, Length Heterogeneity (LH)-PCR was employed to reveal the dominance of *Lactobacillus helveticus* and *Lactobacillus delbrueckii* and, to a lesser extent, *Streptococcus thermophilus* and *Lactobacillus fermentum* (Santarelli et al., 2008). In another instance, the investigation of whey starters was facilitated by reverse-transcription (RT)-PCR amplification of the 16S rRNA transcript (i.e. assessing viable cells only) followed by culture-independent T-RFLP (Terminal Restriction Fragment Length Polymorphisms) analysis, to monitor the population dynamics of the metabolically active fraction in the microbiota of a defined starter over a fermentation period (Sanchez et al., 2006). In this instance T-RFLP was used as a semi-quantitative means of analysis (on the basis of peak area ratios). Ultimately, some differences between the data generated by T-RFLP and colony counting were noted when monitoring *Lactococcus lactis* subsp. *lactis* and *Leuconostoc citreum*. Although, over the first 9 h, both approaches yielded similar results, after 24 h there was a clear difference as T-RFLP detected higher levels of *L. lactis* subsp. *lactis* than were revealed by colony counting, i.e. approximately 90% compared to 80%, respectively. This indicates that a proportion of these cultures remained metabolically active but in a viable but non-cultivable (VNC) state and thus were not detectable by culture-dependent methods (Sanchez et al., 2006). Finally, the bacterial diversity of natural whey cultures has also been investigated

using denaturing high performance liquid chromatography (DHPLC) as well as DGGE, with results comparable to those noted previously in that *Lb. delbrueckii*, *Lb. helveticus*, *S. thermophilus* and *L. lactis* were all detected (Ercolini et al., 2008).

3.3. Evaluation of the microbial diversity of different cheeses

There have been a considerable number of molecular-based studies dedicated to investigating the microbial composition of cheese. Here we divide these investigations into different subcategories i.e. (Section 3.3) a comparison of the microbiota of different cheeses, (Section 3.4) a comparison of the outcomes when these investigations are carried out using culture-dependent and -independent approaches, (Section 3.5) investigations focusing on population dynamics during the fermentation process as well as (Section 3.6) studies that focus on the detection of spoilage related and pathogenic microorganisms.

First we will summarise investigations which have highlighted the large diversity of the cheese-associated microbes, which is itself a reflection of the great diversity in cheese making approaches. The most consistent observation across these studies is the increased microbial diversity apparent in artisanal, relative to industrially manufactured, cheeses, regardless of the type of cheese. This pattern undoubtedly reflects the frequent use of raw milk and undefined starters in artisanal cheeses. Four types of mozzarella cheeses were the focus of attention when DGGE was first employed to investigate a dairy microbial environment (Coppola et al., 2001). These 4 cheeses were made from (a) pasteurised cows milk and commercial starters, (b) raw water-buffalo milk and natural whey cultures, (c) raw cows milk and natural thermophilic milk cultures and (d) raw cows milk without a starter culture, respectively. The analysis of cheeses (a) and (c) led to the detection of *S. thermophilus* only whereas *S. thermophilus*, *L. lactis* and *Lactobacillus* species were detected in cheese (b) and (d). Cheese (d) also contained *Enterococcus faecalis* and *Leuconostoc lactis*, thereby highlighting that the greatest diversity resulted from the use of raw milk and the absence of starter (Coppola et al., 2001). Similar such studies, which again employed a DGGE approach, but which focused on cheese produced from ewes' or goats milk, have also revealed the presence of a more diverse flora in artisanal cheeses (Bonetta et al., 2008; Randazzo et al., 2006) (Table 3). In another instance, the focus turned to the use of SSCP, as well as culturing on brain heart infusion, to assess the impact of pasteurisation on rind development of red-smear soft cheeses (Feurer et al., 2004). This approach revealed bacteria which were common to both cheeses (Table 2), but also highlighted the specific association of *Carnobacterium maltaromaticum*, *L. lactis* subsp. *cremoris*, *Sporanaerobacter acetigenes* and an uncultured Proteobacteria with the pasteurised milk cheese smear whereas the raw milk cheese smear exclusively contained *Corynebacterium casei*, *Lactobacillus curvatus* subsp. *curvatus*, *Marinolactibacillus psychrotolerans*, *Microbacterium gubbeenense*, *Brachybacterium*, *Lactobacillus sakei*, *Pseudoalteromonas* species, and an uncultured *Flavobacteriaceae* within its surface smear (Feurer et al., 2004). Additional observations made by other groups include the examination of four different commercial cheeses (two produced from raw milk and two from pasteurised milk) again revealing a greater diversity of the microbes in the raw milk cheeses (Ogier et al., 2004). This study was also notable as it recorded, for the first time, the presence of *Pseudoalteromonas* and *Halomonas* in a cheese core (Ogier et al., 2004). The ripening conditions employed also have a significant impact on the microbial composition of cheese. This fact was revealed when TTGE fingerprinting and RAPD (Random Amplified Polymorphic DNA) analysis was used to compare the microbiota of two farmhouse cheeses manufactured from raw goats milk in the absence of a starter culture. The key difference related to the fact that the cheeses were ripened as hard (Quesailla Arochena) and soft cheeses (Torta Arochena), respectively (Martin-Platero et al., 2009). Although some species were common to both cheeses, only the hard cheese contained *Hafnia alvei*, *Leuc. lactis* and *Mycobacterium agalactiae* while *Serratia liquefaciens*, *Leucobacter* species and *E. faecalis* were detected in the soft cheese exclusively (Martin-Platero et al., 2009). It is also worth noting that a study comparing the microbial composition of raw and pasteurised milk revealed higher levels of *Enterobacteriaceae*, *Citrobacter* sp. and *Bacillus* species in pasteurised milks whereas raw milk was dominated by *Bifidobacterium* and also contained more *L. lactis*, *S. thermophilus*, *Lactobacillus pentosus*, *Lactobacillus plantarum* and *Corynebacterium afermentans* (Duthoit et al., 2005).

Other culture-independent techniques have also been employed to investigate the microbial composition of a large variety of cheeses. These include techniques such as T-RFLP, which detected staphylococci, microbacteria, brevibacteria and corynebacteria on the Tilsit cheese surface. T-RFLP also revealed the presence of *Carnobacterium* which went undetected when culture-dependent methods were employed (Rademaker et al., 2005). Another technique which has been applied with success to study the microbial composition of cheese is FISH. In one instance this method determined the distribution of LAB in different regions of Stilton cheese (Fig. 2). This study revealed that the cheese core was dominated by *L. lactis*, that the veins and surface were dominated by lactobacilli and *Leuconostoc* and that other unidentified coccoid microorganisms were also detected at lower levels throughout (Ercolini et al., 2003). FISH analysis has also been employed to characterise the yeast population of Livarot cheese surface, revealing that *Candida catenulata* and *Geotrichum* species dominate (Mounier et al., 2009). A similar approach, i.e. fluorescent whole cell hybridisation (FWCH), detected the presence of *Enterococcus italicus*, a recently described dairy-associated enterococcal species, in raw milk cheese (Fornasari et al., 2008).

Table 3
DGGE profile results of bacterial and yeast microflora of artisanal cheeses compared to industrial cheeses (Bonetta et al., 2008).

Microorganisms	Artisanal samples	Industrial samples
Bacteria	*Lactococcus lactis* subsp. *lactis*	*Lactococcus lactis* subsp. *lactis*
	Lactococcus lactis subsp. *cremoris*	*Lactococcus lactis* subsp. *cremoris*
	Streptococcus species	*Streptococcus* species
	Lactococcus garvieae	
	Streptococcus parauberis	
	Streptococcus macedonicus	
Yeasts	*Geotrichum* species	*Geotrichum* species
	Kluyveromyces lactis	*Kluyveromyces lactis*
	Candida sake	*Candida sake*
	Saccharomyces exigus	*Penicillium* species
	Saccharomyces silvae	
	Yarrowia lipolytica	
	Candida catenulate	

3.4. Comparison of outcomes when culture-dependent and culture-independent approaches are used to assess the microbiota of milk and cheese

Culture-independent approaches have a tremendous advantage in that they can reveal microbes that are difficult, or impossible, to culture. While a number of studies, such as that by Van Hoorde et al. (2008), have highlighted this benefit, others have benefited from using parallel culture-dependent and -independent based approaches (Table 2). Indeed, discrepancies with respect to the detection of particular species were noted when different approaches were taken to investigate the microbiota of raw milk Alberquilla cheese i.e. culture-dependent approaches detected *Leuconostoc mesenteroides*, *Leuconostoc pseudomesenteroides* and *Pediococcus urinaequi* while culture-independent TTGE revealed *Lactobacillus acidophilus* and an unidentified nitrogen-fixing bacterium (Abriouel et al., 2008). In a Lebanese artisanal raw goats milk cheese, *Lb. curvatus* and *S. thermophilus* were identified by culture dependent assessment but *Staphylococcus haemolyticus*, *Streptococcus* sp., *Escherichia coli*, *Clostridium bifermentans*

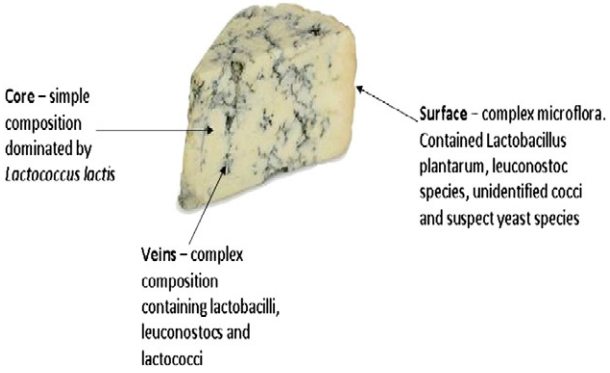

Fig. 2. FISH analysis was able to depict the dominant microorganisms in the different areas of Stilton cheese, i.e. core, surface and veins (Ercolini et al., 2003).

and *Eubacterium tenue* were detected by culture-independent TTGE only (Serhan et al., 2009). A Camembert cheese was found to contain, on the basis of culture-dependent investigations, *Lb. delbrueckii* subsp. *bugaricus* and subsp. *lactis* as well as *Lactobacillus casei* subsp. *casei* but culture-independent TTGE instead revealed the presence of *Lactobacillus paracasei* subsp. *paracasei*. With 16S rRNA gene analysis it can be difficult to distinguish between the *L. casei* and *L. paracasei* which may be responsible for these apparent differences (Henri-Dubernet et al., 2004). In Feta cheese, culturing and DGGE analysis of the bacteria present were inconsistent but corresponding analyses of the yeast population provided comparable results (Rantsiou et al., 2008b) (Table 2). Notably, the culture-independent approaches revealed that a number of thermophilic LAB, including *Lb. plantarum*, *S. thermophilus* and *Lactococcus* species were in a VNC state (Rantsiou et al., 2008b). In raw ewes' milk cheese Pecorino Crotonese, culture-dependent assessment detected the majority of the microbes revealed by culture-independent, TTGE analysis (Table 2), however culture-independent TTGE also revealed a more diverse *Lactobacillus* population which included *Lactobacillus buchneri*, *Lb. fermentum*, *Lb. delbrueckii* and *Lb. plantarum/pentosus* (Randazzo et al., 2009b). It was noted that only culture-independent DGGE succeeded in detecting *Leuc. mesenteroides* in raw goats milk Bukuljac cheese (Nikolic et al., 2008). Culture-independent DGGE assessment also revealed, for the first time, the presence of *S. thermophilus* in the Spanish raw cows milk cheese, Casin (Alegria et al., 2009). In Parmigiano Reggiano cheese, *Pediococcus acidilactici* was detected by culture-dependent approaches only whereas *Lb. fermentum* was exclusively identified by culture-independent (DGGE) (Gala et al., 2008), a study of Saint-Nectaire cheese revealed significant differences between culture-dependent and culture-independent (SSCP) results (Table 2) (Delbes et al., 2007) and, finally, in Salers cheese, culture-dependent methods detected a more diverse yeast population consisting of *Candida intermedia*, *Candida tropicalis*, *Candida rugosa* and *P. guilliermondii* (Callon et al., 2006, 2007). Detailed results of these studies can be found in Table 2.

These observations highlight the benefits of applying a polyphasic approach, i.e. culture-dependent and culture-independent, when assessing a microbial community. However, it is worth noting that in many of the studies where culture-dependent methods highlighted a more diverse microbial composition, these analyses also employed molecular techniques (D/TTGE and SSCP) to then identify the isolates (Delbes et al., 2007; Henri-Dubernet et al., 2008), thus, emphasising our increasing reliance of culture-independent technologies to determine microbial diversity.

3.5. Investigation of microbial composition and succession during cheese manufacture

In addition to facilitating an analysis of the microbiota of milk and cheese diversity, as well as the distribution of microorganisms throughout different regions of a cheese, culture-independent fingerprinting has been used to assess how these microbial populations shift from that present in milk at the beginning of the fermentation process, throughout curd maturation, until the final cheese. The first application of SSCP fingerprinting to obtain such an insight focused on the production of Salers cheese from raw cows milk (Duthoit et al., 2003). In the study the authors detected a highly diverse microbiota, which included a variety of lactic acid bacteria which dominated the microbiota throughout fermentation. The application of SSCP fingerprinting in this study was also notable for the fact that it revealed, for the first time, the presence, of coryneform bacteria, i.e. *Coryneform variabilis*, *Coryneform afermentans*, *Coryneform bovis* and *Coryneform flavescens*, in a cheese core (Duthoit et al., 2003). Another study, which applied DGGE to profile the microbial pattern of Cabrales cheese, a Spanish raw cows milk cheese, revealed that the initial microbiota of the raw milk was remarkably similar to that of the ripened cheeses, with *L. lactis* subsp. *lactis* populations being dominant throughout the process. It was apparent, however, that lactobacilli appeared during ripening. DGGE also detected the presence of low levels of *Bifidobacterium psychroaerophilum* in cheese after 90 days and revealed that the fungal flora changed significantly after day 15 of cheese ripening (Fig. 3) (Florez and Mayo, 2006). Notably, the microflora of Cabrales cheese had previously been assessed using conventional plating methods (Florez et al., 2006). Although a comparison of both studies revealed a broadly similar outcome, it was apparent that *B. psychroaerophilum* was detected by culture-independent approaches only and that this strategy highlighted a more diverse *Lactococcus* population. In a similar study, DGGE analysis was applied to monitor microbial diversity during the production of raw milk, Fontina, cheese. The raw milk was dominated by LAB, which is

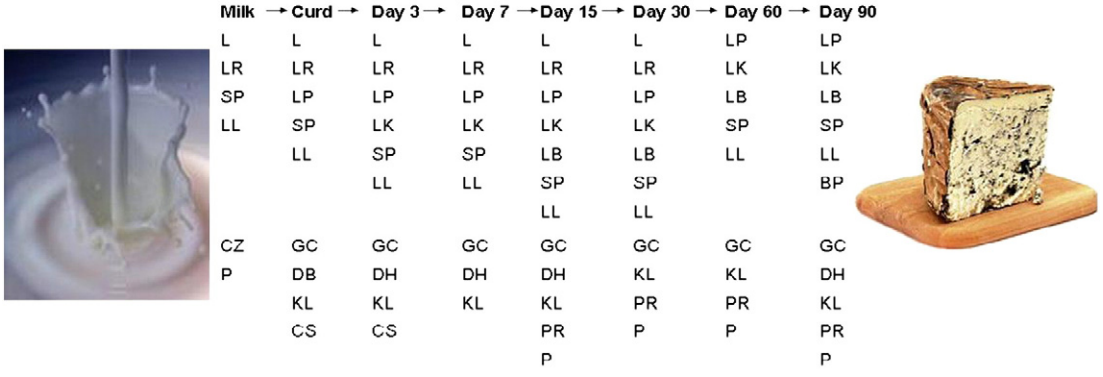

Fig. 3. Microbial changes during the manufacture of artisanal Cabrales cheese monitored by DGGE analysis (Florez and Mayo, 2006). L- *Lactococcus lactis*, LR- *Lactococcus raffinolactis*, SP- *Streptococcus parauberis*, LL- *Lactococcus lactis* subsp. *lactis*, CZ- *Candida zeylanoides*, P- *Penicillium chrysogenum/griseofulvum*, LP- *Lactobacillus plantarum*, GC- *Geotrichum candidum*, DH- *Debaryomyces hansenii*, KL- *Kluyveromyces lactis*, CS- *Candida silvae*, LK- *Lactobacillus kefiri*, LB- *Lactobacillus buchneri*, PR- *Penicillium roqueforti*, BP- *Bifidobacterium psychrophilum*.

consistent with previous culture-based studies (Senini et al., 1997), but also contained *Staphylococcus* sp., *Pantoea sp.*, *Chryseobacterium* and *Moraxella*. After heating of the milk (47–48 °C) to form curds the bacterial population became more homogenous with *S. thermophilus* and enterococci dominating the fresh curd population. These changes also coincided with the appearance of *Kocuria rhizophila* and *Klebsiella oxytoca*, an increase in *Pantoea* sp. and a decrease in *Chryseobacterium*, *Moraxella*, and *Macrococcus* sp. (Giannino et al., 2009).

While the comparisons referred to in the previous paragraph were possible due to the availability of data from related culture-based studies, other studies have employed traditional culturing approaches in parallel with modern culture-independent assessments to gain further insight into the complex microbial changes that occur during dairy fermentations. Ercolini et al. have monitored the microbiota of whole raw buffalo milk and the changes which occur during the production of traditional water buffalo mozzarella cheese using PCR-DGGE and culturing. The DGGE fingerprint revealed that the milk microbiota changes after starter addition, in the form of a natural whey culture (NWC), and the cheese microbiota remained similar to the NWC from its addition until the final cheese product was generated. The parallel culture-based approach established that buffalo milk is rich in LAB, with an increase in both thermophilic and mesophilic microorganisms occurring after starter addition (Ercolini et al., 2001, 2004). Another such study, employing culture-based and DGGE fingerprinting analysis of a raw cows milk cheese, detected a diverse LAB population present from raw milk to the final cheese (Randazzo et al., 2002). Furthermore, RNA was extracted from milk and cheese and assessed by RT-PCR-DGGE. Although the results of RNA- and DNA-generated patterns were comparable, differences existed in that the RNA-derived results showed that *L. lactis* and *Leuc. mesenteroides* were not as metabolically active in milk and curd as had been anticipated whereas *Lb. acidophilus* and *Lb. delbrueckii* subsp. *bulgaricus* were particularly active (Randazzo et al., 2002).

A raw cows milk cheese, Castelmagno, which was manufactured without the addition of starter cultures during summer, was also assessed by both DGGE and culturing methods, again revealing a good correlation between culture-dependent and -independent methods (Dolci et al., 2008). DGGE revealed a predominance of *L. lactis* subsp. *lactis* and subsp. *cremoris* throughout both the cheese making and ripening stages. DGGE also more effectively detected *Lb. plantarum*, *Macrococcus caseolyticus* and *Streptococcus agalactiae* during the early stages and *Lactobacillus kefiranofaciens* from the latter stages of the process (Table 2). This information was supplemented by culture-derived results which established the presence of lactobacilli throughout the cheese making and ripening process (Dolci et al., 2008). Interestingly, Castelmagno that had been produced in winter was subsequently assessed using the same approach but in this instance an RNA-based investigation was also carried out (Dolci et al., 2010). The culture-based approach established that lactococci and lactobacilli increased from milk to the curd up to day 30 of ripening after which time they began to decrease. In contrast, DNA and RNA fingerprinting determined that *L. lactis* dominated, and continued to be metabolically active, until the end of ripening. In addition to these specific outcomes, it is important to note that a seasonal influence on microbial population levels was also apparent (Dolci et al., 2010).

Combinations of conventional and culture-independent (DGGE) approaches were used to study the lactic acid bacteria of raw cows milk cheese, Provolone del Monaco (Aponte et al., 2008). All approaches revealed that *S. thermophilus* and *Streptococcus macedonicus*, and to a lesser extent enterococci, dominated throughout the manufacturing process. An increase in lactobacilli populations was detected during ripening, although it was noted that *Lb. delbrueckii* was detected by DGGE only (Table 2). Interestingly, a comparison of three different DNA extraction protocols highlighted the importance of using a suitable extraction method (Aponte et al., 2008). A Croatian raw sheep's milk cheese has also been assessed by culture dependent and independent methods (Fuka et al., 2010). DGGE analysis revealed a more diverse microbial composition, detecting the presence of *E. faecium/faecalis*, *L. lactis* subsp. *lactis*, *Enterobacter cancerogenus*, *Klebsiella terrigena*, *Klebsiella pneumoniae*, *Pantoea agglomerans*, *Entrobacter hormaechei*, *Staphylococcus equorum*, *Staphylococcus sciuri*, *Staphylococcus gallinarum*, *Staphylococcus saprophyticus* and non-fermentative *Acinetobacter* in milk, with *M. caseolyticus* and *Pseudomonas fragi* being sporadically identified in milk and the fresh curd. After 30, 90 and 120 days ripening, the dominant species was *L. lactis* subsp. *lactis* with the presence of *Lb. curvatus*, *S. saprophyticus* and *Enterococcus* species also being noted (Fuka et al., 2010). Alessandria et al. (2010) evaluated the dominant population of an artisanal cheese produced on the Cape Verde Islands. Here, the authors employed culture-dependent methods, alongside DNA and RNA culture-independent PCR-DGGE methods. Overall, the culturing and DGGE profiling revealed similar results (Table 2). However, a number of bacteria were only detected by the RNA-based strategy. These included *Moraxella oslonesis*, *Lb. helveticus*, *Leuc. pseudomesenteroides* and *K. rhizophila* (Alessandria et al., 2010). Finally, the use of DGGE by Randazzo et al. (2010) has highlighted the diversity of the LAB population in Pecorino Crotonese cheese. *L. lactis* subsp. *lactis* dominated throughout the process with *Lactobacillus brevis* and *Lb. buchneri* also being detected. During ripening a number of other species, including *Lb. plantarum/pentosus*, *Lb. fermentum*, *Leuc. mesenteroides*, *Lb. delbrueckii* and *Lactobacillus rhamnosus* were identified, and it was revealed that *S. thermophilus* became dominant at this point. On the basis of the appearance and disappearance of bands, the authors were able to assess the impact the microbial composition on flavour development. It was apparent that when LAB species were abundant in the final product there were higher concentrations of volatile compounds which contribute floral and fruity notes (Randazzo et al., 2010).

3.6. Application of molecular biology for pathogen and spoilage related investigations of milk and cheese products

DNA-based technologies benefit from being capable of providing a rapid assessment of the composition of a microbial niche. This is particularly important when determining the presence of pathogenic microbes in dairy products. Quantitative PCR has been particularly beneficial here allowing the rapid identification and quantification of such microorganisms. A number of studies have developed and validated qPCR methods to monitor pathogens such as *Mycobacterium avium* subsp. *paratuberculosis* (MAP), *Staphylococcus aureus* and *Listeria monocytogenes* (Slana et al., 2008; Graber et al., 2007; Rantsiou et al., 2008a). MAP, the causative agent of Johnes disease in cows and which has, on occasion, been associated with Crohn's disease in humans (Ayele et al., 2001), is a problem for the dairy industry. Because of concern and debate regarding the possibility that milk may serve as a vehicle for the transmission of MAP to humans, the rapid detection of MAP in milk and dairy products is of key importance to dairy microbiologists (Hermon-Taylor and Bull, 2002). Culturing of this microbe is long and laborious, often taking months and with no guarantee of success. In a study of 345 milk samples, the culture-dependent approach, which was conducted over 8 months, failed to detect MAP, however 111 of the samples were found to be positive when an alternative, rapid qPCR-based approach was taken (Slana et al., 2008). Similar patterns have been observed in subsequent studies of milk and cheese (Botsaris et al., 2010; Slana et al., 2009). In each case qPCR was capable of detecting MAP at levels below 10 cells per ml of milk. While these studies highlight the importance of a culture-independent approach, it should also be noted that the detection of MAP by these methods were DNA-based and that a RNA approach could also be conducted to reveal if the MAP in question are dead DNA or in a viable but non-cultivable state.

Another microorganism of interest to the dairy industry is *S. aureus*, the primary cause of mastitis infection in cows and a significant food pathogen. Graber et al. (2007) devised a qPCR method which facilitated the rapid detection of *S. aureus* in bovine milk at low concentrations (1–10 CFU per ml; i.e. 50 times more sensitive than plating). The benefits of employing qPCR to detect *S. aureus* in milk have also been highlighted in

other studies (Studer et al., 2008). While a broad variety of target specific DNA-based approaches, including qPCR, are available to detect the presence of pathogens, a community-based analysis can also be revealing. Delbes and Montel (2005) used SSCP analysis in order to detect and discriminate between individual components of the staphylococcal population of a raw cows milk cheese. This analysis detected the presence *S. aureus*, *S. equorum* and *S. saprophyticus* and revealed the extent to which levels of these species varied during the cheese-making process, with major increases in *S. aureus* from raw milk to the 12 h cheese being particularly notable (Delbes and Montel, 2005). Similarly, although the analysis of raw milk produced by four mastitic cows, by PCR-DGGE revealed the presence of a number of pathogens known to cause infection, i.e. *Escherichia* sp., *Enterobacter* sp., *S. aureus* and *Streptococcus uberis*, the corresponding use of three types of selective agar (Blood, TKT and MacConkey agar) incorrectly indicated that coliforms were the sole cause of infection (Kuang et al., 2009). The accurate identification of the aetiological agent is obviously of key importance with respect to the treatment of mastitis and, thus, in this instance, the risks associated with relying solely on culture-generated information are apparent.

Another pathogen which is of major concern for the dairy industry is *L. monocytogenes*, with soft, raw milk cheeses being particularly problematic. Thus, rapid and accurate detection of this pathogen is critical. Rantsiou et al. (2008a) devised a *L. monocytogenes*-specific qPCR method which was employed to test 33 fresh cheese and 11 ripened cheese samples made from raw goats milk. This approach revealed that 4 fresh cheese samples were positive for *L. monocytogenes*, a number which increased to 8 after the inclusion of an enrichment step (Rantsiou et al., 2008a). SSCP fingerprinting of raw milk cheese has also been employed to provide an insight into the microbes that may inhibit *L. monocytogenes* in cheese (Saubusse et al., 2007). Two cheese groups were examined with group I containing high numbers of *L. monocytogenes* whereas the counts in cheeses from group II were low. SSCP analysis of the microbial populations revealed that group II had a greater number of peaks corresponding to *Enterococcus faecium*/*saccharominimus*, *Chryseobacterium* sp./*flavescens* and *Lactococcus gravieae*/*lactis* compared to group I. To determine if these species were influencing the presence of *Listeria* in these cheeses, a pasteurised milk cheese model, into which *L. monocytogenes* and the putative inhibiting strains were introduced, was manufactured. Following testing, it was determined that inhibition occurred when *L. lactis* and *L. garvieae*, and to a lesser extent *E. saccharominimus* and *C. flavescens*, were included. Strangely, neither the production of inhibitory compounds, such as bacteriocins, nor a drop in pH seemed responsible, and thus the mechanism via which the pathogen was inhibited was not apparent (Saubusse et al., 2007). This study reveals how culture-independent methods can be applied, to a complex microbial community, to reveal the presence of microorganisms with possible antimicrobial properties.

Clostridia, representatives of which can cause the defect late blowing in cheese, are responsible for massive financial losses in the dairy industry. Detection of these spoilage microorganisms is therefore extremely important. In one instance, seventeen raw milk cheeses were subjected to DGGE-based analysis to characterise the clostridial population, leading to the identification of four species i.e. *Clostridium butyricum*, *Clostridium beijerinckii*, *Clostridium sporogenes* and *Clostridium tyrobutyricum* (Cocolin et al., 2004). The clostridial population of twenty raw milk cheeses was the subject of another investigation employing TTGE fingerprinting. Four different species of clostridia, i.e. *C. tyrobutyricum*, *C. beijerinckii*, *C. butyricum* and *C. sporogenes*, were detected by TTGE, which contrasted with the identification of only two species, *C. sporogenes* and *C. tyrobutricum*, when a culture-based approach was employed (Le Bourhis et al., 2005).

SSCP fingerprinting has also been used to monitor how milking practice and farm hygiene influence the microbial composition of milk (Verdier-Metz et al., 2009). Milk samples collected from dairy farms, after morning milking, were analysed. Milking practices were monitored by surveying the hygiene process applied to udders and the milking system, pre- and post-milking. The samples were divided into three groups on the basis of milking practices and the associated microbiota, as determined by SSCP; Group A being those where hygiene practices were most intensive while Group C was least hygienic. While milk from all three groups contained *L. lactis* at equal concentrations (Fig. 4), the samples differed with respect to their overall microbial diversity. This diversity was measured by the Shannon Index and it was revealed that Group A had the lowest diversity (0.88) while Group B (1.09) and Group C (1.18) had higher diversity (Verdier-Metz et al., 2009). Other studies have used these approaches to monitor the impact of storage conditions. Lafarge et al. (2004) revealed that *L. lactis*, followed by *Staphylococcus* species, *K. pneumoniae* and, to a lesser extent, *Listeria*, *Enterococcus* and *Streptococcus* species, were the major raw milk species present prior to refrigeration. After refrigeration at 4 °C for 24 h, many of the species initially identified were still present but their relative proportions were clearly altered (Table 4). Unsurprisingly, there were increases in

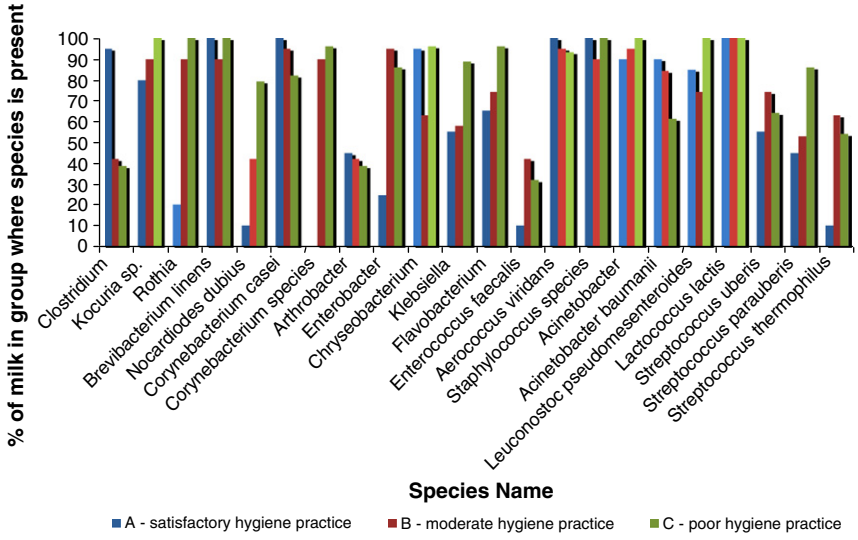

Fig. 4. SSCP peak assignment. Peaks are assigned based in the percentage of milks in each group where a specific peak is present representing a bacterial species. The percentage is based on the number of milk samples in a group, Group A = 20; Group B = 19; Group C = 28. Developed from results of study conducted by Verdier-Metz et al. (2009).

the numbers of *Listeria*, which are psychrotrophs (Lafarge et al., 2004). This approach was deemed more sensitive than traditional methods which indicated that incubation for 48 h at 4 °C was required before a bloom in the psychrotrophic microbiota occurred (Brouillaud-Delattre et al., 1997). Culture-independent, real-time qPCR has also been applied successfully to investigate the impact of an even greater variety of factors i.e. thermisation, carbon dioxide (CO_2) and microfiltration at 4 °C and 8 °C. The investigation revealed that while levels of all species increased when milk was stored at 8 °C, *Acinetobacter calcoaceticus*, *Aerococcus viridans*, *S. aureus*, *S. uberis* and *Corynebacterium* were stable for 7 days at 4 °C, regardless of the treatments employed. However, *P. fluorescens* differed in that levels in raw milk and CO_2 treated milk increased over 7 days relative to untreated controls (Rasolofo et al., 2010).

4. Conclusion and future prospects

Since the invention of PCR technology, the field of microbial ecology has evolved with tremendous speed and molecular methods are continuing to revolutionise our understanding of the composition and population dynamics of microbial communities in various environments. The culture-independent methods described in this review have facilitated substantial progress in food microbial ecology by facilitating the simultaneous study of viable, non-cultivable and stressed/injured microbes. Significant advances have included the identification, for the first time, of various microorganisms from milk and cheese, such as coryneform bacteria, *Pseudoalteromonas* and *Halomonas* in the cheese core and *S. thermophilus* in Spanish cheese. Other studies have highlighted the ability of culture-independent methods to rapidly identify microbes of interest to the food industry, including MAP, *S. aureus* and clostridia. While many publications have highlighted the benefits of using polyphasic approaches, i.e. both culture-dependent and culture-independent strategies, it was noted that culture-independent methods were more rapid, sensitive and less susceptible to bias than culture-dependent methods. In addition, the ability to distinguish between dead or viable cells is an important factor in understanding the process of cheese manufacture. Upon lysis, cells release their intracellular components which contribute to cheese development, especially in the context of contributing to the textural, flavour and aroma development in a cheese. However, many cells which appear to be dying on the basis of conventional culturing, may be in a permeabilised but viable state and therefore the ability to distinguish between dead and viable cells, using a combination of DNA- and RNA-based approaches, is another important benefit of culture-independent methods. It is thus apparent that culture-independent methods have been of critical importance with respect to our investigation of the microbial community of raw milk and raw milk cheese and our development of a better understanding of the role of these microbes in the flavour, quality and safety of dairy products.

Table 4
Changes observed in milk microbiota during refrigeration at 4 °C for 24 h, monitored by band intensity from DGGE/TTGE profile of one milk sample (Lafarge et al., 2004).

Bacteria detected	Milk + 24 h refrigeration
Lactococcus lactis	−
Lactobacillus sp.	D
Listeria sp.	+
Pseudomonas sp.	+
Streptococcus uberis	+
Klebsiella pneumoniae	−
Escherichia coli	D
Enterobacter sp.	−
Serratia marcescens	=
Brevibacterium linens	D
Propionibacterium acidipropionici	−
Staphylococcus sp.	+
Kocuria sp.	−
Propionibacterium jensenii/thoenii	A

A, appearance; D, disappearance; + increased intensity; −, decreased intensity; =, same intensity.

While the techniques described here have been vital to our understanding of dairy microbiology, the field of microbial ecology is constantly evolving. The last few years have seen the introduction of next-generation sequencing technologies which are replacing other culture-independent approaches. These technologies provide the benefits of reduced labour time, lower reaction volumes, extended number of sequence reads as well as high-throughput sampling. This has proven extremely successful in profiling the microbiota of various environments including deep sea vents, gut and soil amongst other environments. However, there have been extremely few studies in which these technologies have been utilised to investigate the microbial composition of foods. Those which have taken place have revealed the diverse microbial populations present in fermented seafood (Roh et al., 2010), vegetables (Jeon et al., 2011) and rice bran (Nakayama et al., 2011). Notably, such an approach has also been applied recently to a raw milk cheese (Masoud et al., 2011) revealing a diverse subdominant population which went undetected by DGGE analysis. These studies suggest that the application of next generation DNA sequencing technologies will greatly enhance our research in the area of food microbiology, including that of raw milk and raw milk cheeses.

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