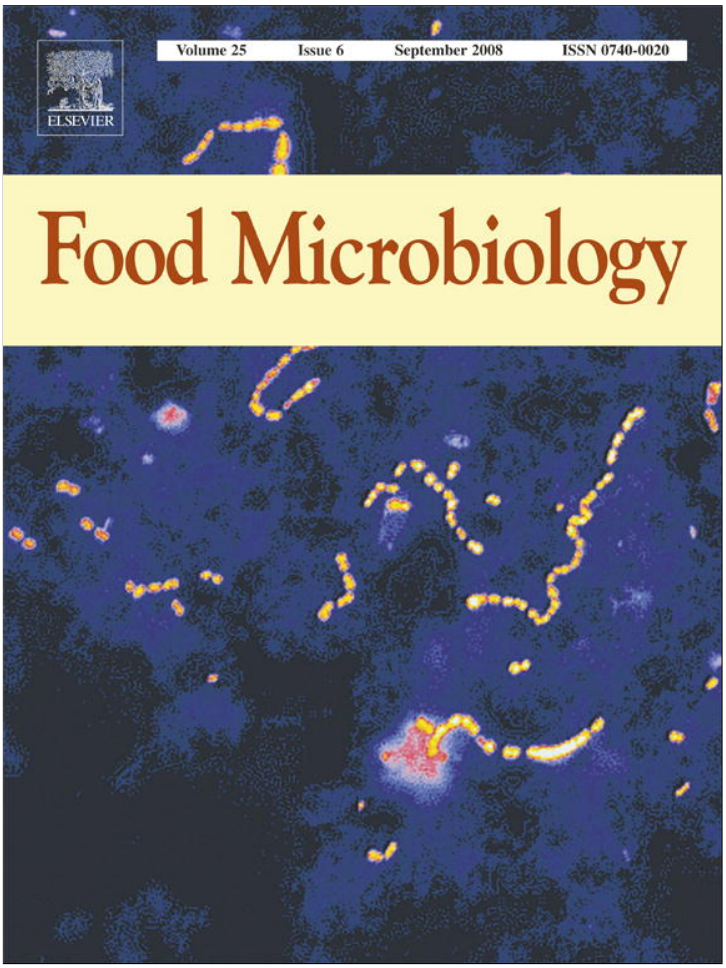

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Review

Recent advances in molecular techniques to study microbial communities in food-associated matrices and processes

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ABSTRACT

In the last two decades major changes have occurred in how microbial ecologists study microbial communities. Limitations associated with traditional culture-based methods have pushed for the development of culture-independent techniques, which are primarily based on the analysis of nucleic acids. These methods are now increasingly applied in food microbiology as well. This review presents an overview of current community profiling techniques with their (potential) applications in food and food-related ecosystems. We critically assessed both the power and limitations of these techniques and present recent advances in the field of food microbiology attained by their application. It is unlikely that a single approach will be universally applicable for analyzing microbial communities in unknown matrices. However, when screening samples for well-defined species or functions, techniques such as DNA arrays and real-time PCR have the potential to overtake current culture-based methods. Most importantly, molecular methods will allow us to surpass our current culturing limitations, thus revealing the extent and importance of the 'non-culturable' microbial flora that occurs in food matrices and production.

Contents

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1. Introduction

Knowledge about microbial community composition, its different populations and its potential interactions in food-associated matrices is crucial to ensure production of safe and high-quality food. Traditionally, the occurrence of microorganisms in a given environment or in an industrial process has been studied by culture-based methods. Nevertheless, it is well recognized that these methods often fail to characterize (minor) populations or microorganisms for which selective enrichment is necessary. Moreover, stressed or weakened cells often need specific culture conditions to recover and to become cultivable. In addition, conventional methods are not able to detect non-culturable cells, representing a serious drawback of these methods. Also in relatively simple food matrices, such as fermented foods in which 'culturable' microorganisms generally predominate, Ampe et al. (1999) demonstrated that at least 25–50% of the active microbial community could not be cultured *in vitro*. These limitations have prompted the development of culture-independent techniques of which those based on polymerase chain reaction (PCR) amplification and detection of nucleic acids are the most predominant (Yang et al., 2001). Compared to conventional methods, molecular methods are generally faster, more specific, more sensitive and more accurate, allowing a precise study of the microbial populations and their diversity in given ecosystems.

The importance of ecological concepts for understanding the presence and growth of microorganisms is well recognized by food microbiologists (Giraffa and Neviani, 2001; Giraffa, 2004). Flavor, texture and preservative qualities of many food products like e.g. cheese, yogurt, sausages, wine and sour dough breads are established through (the use of) different microbial communities (Delfini and Formica, 2001; Duthoit et al., 2003; Willems et al., 2003; Temmerman et al., 2004). Close monitoring of the changes in microbial populations throughout the production process allows better comprehension and management of the microbial processes involved in food processing and ripening (Barriga et al., 1991; Walls and Scott, 1997; Delfini and Formica, 2001) and improvement of microbiological safety by fast and accurate monitoring of potential pathogens (Liu, 2004). Altogether, accurate detection and reliable identification at the species (and strain) level, as well as ecological studies on the community composition and dynamics, support food quality and safety as requested by consumer, industry and government. However, unlike ecosystems such as soils, sediments or aquatic environments, most molecular microbial community analysis techniques have thus far only been used infrequently for microbial characterization of food and food-related matrices, except for denaturing gradient gel electrophoresis (DGGE) (Lopez et al., 2003; Ercolini, 2004; Rantsiou et al., 2005; Florez and Mayo, 2006; Hovda et al., 2007a, b). Nevertheless, these methods are increasingly implemented in the food industry as well (e.g. Cambon-Bonavita et al., 2001; Rudi et al., 2002; Cardinale et al., 2004; Kim and Chun, 2005; Delbès et al., 2007; Hovda et al., 2007a, b; Nordstrom et al., 2007; Justé et al., 2008b), which is confirmed for food fermentations by an excellent book review (Cocolin and Ercolini, 2008).

In this review, we outline some recent advances in the use of molecular PCR-based methods for thorough characterization of microbial communities with (potential) applications to study microbial communities in food, focusing on three main features encompassing microbial diversity, identity and quantity. Whereas studying the microbial diversity in a community allows a fair comparison between different samples, environments or situations, in many cases it is desirable to also know the identity of the microorganisms that are present in one sample but not in another. In addition, quantification is getting increasing attention, e.g. to determine if a certain quantity is expected to be harmful in the case of pathogens or beneficial in the case of probiotics. Advantages, limitations and current applications of these methods are presented in relation to these three criteria.

2. Choice of target genes

Since its introduction in the mid-1980s, PCR has become a fundamental aspect of molecular ecology, and several techniques based on PCR have been developed since then to study microbial communities. In general, DNA serves as a template for PCR amplification of genetic targets with universal (non-discriminative) primers to amplify all target sequences of a given population. As the resulting PCR products amenable for profiling are often of similar size, differentiation must be achieved on the basis of nucleotide sequence differences. Therefore, a crucial step for molecular assessment of microbial communities is the selection of a gene or genetic marker that can be used to differentiate a wide variety of organisms. Suitable genetic targets should have both variable and conserved regions, in which the variable domains, allowing discrimination over a wide range of taxonomic levels, are flanked by conserved sequences, which serve as annealing sites for universal PCR primers. In the absence of highly conserved regions, domains with a lower degree of conservation can be selected as annealing sites for degenerate primers. In general, ubiquitously conserved genes reflecting phylogenetic differences have been used as the primary target in molecular ecology. Alternatively, sequence differences within functional genes may be exploited to study functional diversity.

2.1. Ubiquitously conserved genes

At present, the bacterial ribosomal RNA (rRNA) operon, encompassing a 16S rRNA and 23S rRNA gene as well as an intergenic spacer (IGS) region, is most frequently used as

molecular marker in microbial ecology because of a number of reasons including (i) its universal abundance, (ii) its evolutionary and phylogenetic properties, reflected by the presence of both variable and highly conserved sequence domains, (iii) its high discriminatory potential, (iv) its, often, multiple-copy nature, resulting in more sensitive analyses, and (v) the extensive availability of sequences in public databases such as GenBank (Benson et al., 2004), which enables in turn an accurate description of the microbial populations present in a community (Chakravorty et al., 2007). 16S ribosomal and intergenic spacer sequences are available in public databases of, for example, the 'Ribosomal Database Project' (RDP) (Olsen et al., 1992; Cole et al., 2005) and IWoCS, software to study IGS sequences by a Word Count-based System (D'Auria et al., 2006), respectively, of which the first one currently has over 85,500,730 entries and is monthly updated. Nevertheless, ribosomal sequences do not always reflect sufficient sequence variation to discriminate between particular closely related, but ecologically distinct, taxa. Indeed, ecologically distinct taxa may have had time to accumulate neutral sequence divergence at rapidly evolving loci but not yet at the 16S rRNA level (Fox et al., 1992; Palys et al., 2000). Palys et al. (1997, 2000) demonstrated that DNA sequences of for example protein-coding genes are more effective than the 16S rRNA gene for studying the ecological diversity of certain bacteria in closely related communities. Furthermore, since different copies within a single genome can differ in sequence, multiple ribotypes may be obtained for a single organism, complicating interpretation of the analysis (Dahllöf et al., 2000). Therefore, other housekeeping genes displaying intertaxa sequence variation are becoming more intensively studied, including the gyrase B (*gyrB)* gene (Wang et al., 2007), the elongation factor Tu (*tuf)* genes (Ventura et al., 2003), the dnaK gene (Stępkowski et al., 2003), the RNA polymerase B (*rpoB*) gene (Dahllöf et al., 2000; Giacomazzi et al., 2004; Case et al., 2007), and recA gene families (Rossi et al., 2006). However, in comparison with the 16S ribosomal sequences, databases for these alternative genes only contain a small number of sequences (D'Auria et al., 2006), limiting their use in current microbial ecology.

2.2. Functional genes

Different microbial communities may be composed of quite different groups of species yet in essence perform the same processes. When a specific microbial process is of interest, the functional diversity in a given environment may be monitored by assessing the diversity of so-called 'functional genes', encoding key enzymes in the process of interest, and the identification of predominant gene polymorphisms.

In general, analysis of functional diversity has been correlated with well-characterized processes in soils or aquatic environments such as nitrogen cycling (Wu et al., 2001; Wolsing and Priemé, 2004), sulfate reduction (Geets et al., 2006), and degradation of polycyclic aromatic hydrocarbon compounds (Uyttebroeck et al., 2006). To our knowledge, so far functional gene diversity has not been studied in food-associated matrices. However, several applications of gene functional analysis have been reported for detection of and simultaneous discrimination among food-borne human pathogens (Chizhikov et al., 2001; Volokhov et al., 2002; Chiu et al., 2005).

3. Microbial community analysis techniques

In general, microbial communities can be characterized based on three fundamental properties encompassing diversity, identity and quantity. Although most of the molecular techniques currently used for microbial community analyses each reveal a considerable view on the community, none of them presents the whole picture. In general, there are two kinds of approaches to study microbiology by PCR-based methods. Universal primers of various taxonomic resolutions are used to generate a broad mix of amplicons that are further analyzed through a range of techniques. Alternatively, (functional) group-specific PCR reactions are performed to detect and/or quantify specific organisms or genes of interest. The most predominant of these techniques used in microbial ecology are discussed below. In Table 1 the different applications in food that are referred to in the text are further dissected.

3.1. Microbial diversity

Several well-characterized molecular techniques including denaturing and temperature gradient gel electrophoresis (DGGE/TGGE), single-strand conformation polymorphism (SSCP), terminal restriction fragment length polymorphism (T-RFLP), amplified ribosomal DNA restriction analysis (ARDRA) and (automated) ribosomal intergenic spacer analysis ((A)RISA), have been widely adopted for genetic analysis of microbial communities because they provide a relatively comprehensive description of a given community. More in particular, these techniques are extremely suitable to compare microbial community compositions between different treatments, environments or situations.

3.1.1. Denaturing gradient gel electrophoresis and temperature gradient gel electrophoresis

Both denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) examine microbial diversity based upon electrophoresis of small PCR-amplified DNA fragments (200–700 bp) on an acrylamide gel having a low to high denaturant gradient (Muyzer et al., 1993; Muyzer and Smalla, 1998). Whereas DGGE uses denaturing chemicals such as formamide and urea, a temperature gradient is applied in TGGE. As a result, DNA fragments of similar length but with different sequences can be separated according to their melting properties. Generally, the melting behavior depends on the length of the product, its GC-content and the nucleotide sequence. Initially the melting process is partial, with discrete domains becoming single-stranded, decreasing the mobility of the DNA fragments through the gel. Eventually, strand separation stretches over the entire length of the product with the exception of a GC-rich clamp (40–45 base GC-rich sequence), which is attached to the 5′-end of the forward primer (Sheffield et al., 1989). This clamp is highly stable and holds the strands partially together leading to a molecule whose migration is extremely retarded. Although the gradients might have to be adjusted to a specific sample for optimal resolution (Ogier et al., 2002), these methods have the theoretical potential to detect differences of as little as a single or a few base pairs.

DGGE/TGGE has been powerful to compare (structural changes in) microbial communities as well as for monitoring population dynamics (Sun et al., 2004; Camu et al., 2007). Several regions of the 16S rRNA gene have been used for DGGE or TGGE fingerprinting (Ercolini et al., 2003; Randazzo et al., 2002). However, the length and species-specific heterogeneity of the V3 region within this gene make this region one of the better choices (Florez and Mayo, 2006). In addition, increasingly DGGE profiles of mRNA-derived PCR products are generated to describe the diversity in metabolically active populations (Otawa et al., 2006; Dar et al., 2007). Major advantages of both DGGE and TGGE are their affordability for molecular laboratories and the relatively easy interpretation of results. Furthermore, individual bands can

Table 1
Overview of microbial community analyses in food that are referred to in the text

Technique	Primer	Primer sequence 5′–3′	Gene	Application matrix in this review	Reference
ARDRA	F 27F R 492R	AGAGTTTGATCMTGGCTCAG TACGGYTACCTTGTTACGACTT	16S rRNA gene	Maple sap[a]	Lagacé et al. (2004)
ARISA	F 16S rRNA gene/p2 R 23S rRNA gene/p10	CTTGTACACACCGCCCGTC CCTTTCCCTCACGGTACTG	IGS	Meat, fish, poultry, cheese, smoked salmon[a]	Kabadjova et al. (2002)
ARISA	F 16S rRNA gene/p4 R 23S rRNA gene/p7	GCTGGATCACCTCCTTTCT- GGTACTTAGATGTTTCAGTTC	IGS	Meat, fish, poultry, cheese, smoked salmon[a]	Kabadjova et al. (2002)
ARISA	F 1406f R 23Sr	TGYACACACCGCCCGT GGGTTBCCCCATTCRG	IGS	Goat milk and ensiled corn	Cardinale et al. (2004)
ARISA	F IGSF R IGSReub	GTCGTAACAAGGTAGCCGTA GCCAAGGCATCCACC	IGS	Goat milk and ensiled corn	Cardinale et al. (2004)
ARISA	F S-D-Bact-1522-b-S-20 R L-D-Bact-132-a-A-18	TGCGGCTGGATCCCCTCCTT CCGGGTTTCCCCATTCGG	IGS	Goat milk and ensiled corn	Cardinale et al. (2004)
ARISA	F ISRf R ISRr	GAAGTCGTAACAAGGT CAAGGCATTCACCAT	IGS	Sourdough[a]	De Angelis et al. (2007)
Cloning	F W02 R W18	GNTACCTTGTTACGACTT GAGTTTGATCMTGGCTCAG	16S rRNA gene	Cheese, raw milk	Duthoit et al. (2003)
Cloning	F fD1 R rD1	AGAGTTTGATCCTGGCTCAG AAGGAGGTGATCCAGCC	16S rRNA gene	Pozol	Escalante et al. (2001)
Cloning	F R	TTCCGGTTGATCC[C,T]GCCGGA [C,T]CCGGCGTTGA[A,C]TCCAATT	16S rRNA gene	Kimchi	Kim and Chun (2005)
DGGE	F 338f R 518r	ACTCCTACGGGAGGCAGCAG ATTACCGCGGCTGCTGG	16S rRNA gene, V3	Pozol balls, wine	Ampe et al. (1999), Lopez et al. (2003)
DGGE	F HDA1 R HDA2	ACTCCTACGGGAGGCAGCAGT GTATTACCGCGGCTGCTGGCAC	16S rRNA gene, V3	Dairy products	Ogier et al. (2004), Lafarge et al. (2004)
DGGE	F 63F R 518r	CAGGCCTAACACATGCAAGTC ATTACCGCGGCTGCTGG	16S rRNA gene, V1-V3	Mineral water	Dewettinck et al. (2001)
DGGE	F WBAC1 R WBAC2	GTCGTCAGCTCGTGTCGTGAGA CCCGGGAACGTATTCACCGCG	16S rRNA gene, V7-V8	Wine	Lopez et al. (2003)
DGGE	F U968 R L1401	ACGGGGGGAACGCGAAGAACCTTAC GCGTGTGTACAAGACCC	16S rRNA gene, V6-V8	Beef	Ercolini et al. (2006)
DGGE	F rpoB_1675 R rpoB_2063	TGY CCG ATY GAA ACA CCK GAR GG TGA CGY TGC ATG TTC GMN CCC AT	rpoB gene	Cold-smoked salmon[a]	Giacomazzi et al. (2004)
DGGE	F rpoB_1675ndg R rpoB_2063ndg	TG TCC GAT CGA AAC ACC TGA AGG TGA CGT TGC ATG TTC GCA CCC AT	rpoB gene	Cold-smoked salmon[a]	Giacomazzi et al. (2004)
DGGE	F 341F R 985R	CCTACGGGAGGCAGCAG GTAAGGTTCTTCGCGTT	16S rRNA gene, V3-V6	Salad	Handschur et al. (2005)
DGGE	F DG74f R RW01r	AGGAGGTGATCCAACCGC AACTGGAGGAGGGTGGGGAT	16S rRNA gene	Salad	Handschur et al. (2005)
DGGE	F 338f R 518R	ACTCCTACGGGAGGCAGCAG ATTACCGCGGCTGCTGG	16S rRNA gene, V3	Atlantic Cod (*Gadus morhua*)	Hovda et al. (2007a, b)
DGGE	F 357F R 518R	TACGGGAGGCAGCAG ATTACCGCGGCTGCTGG	16S rRNA gene, V3	Rennet extracts, milk and cheese samples from blue-veinded Cabrales cheese	Florez and Mayo (2006)
DGGE	F RpoF R RpoR	AACATCGGTTTGATCAAC CGTTGCATGTTGGTACCCAT	rpoB gene	Sourdough[a]	De Angelis et al. (2007)
DNA array	KR1 KR2	TGG CTC AGA TTG AAC GCT GGC GGC TAC CTT GTT ACG ACT TCA CCC CA	16S rRNA gene	Ready to eat vegetable salads in modified atmosphere	Rudi et al. (2002)
DNA array	27F 1492R	AGAGTTTGATCCTGGCTCAG TACGG(C/T)TACCTTGTTACGACTT	16S rRNA gene	Sugar thick juice	Justé et al. (2008b)
DNA array		TGGCTCAGATTGAACGCTGGCGGC TGGACCGTGTCTCAGTTCCAGTGTG	16S rRNA gene	Theoretical development on pure strains array is now ready for the food industry	Eom et al. (2007)
FAME	/	/		Biofilms from breweries	Timke et al. (2005a)
FISH	/	/	16S rRNA gene	Wine	Sohier and Lonvaud-Funel (1998)
FISH	lm3	CGGGTGCTICCCACTTTCATG	16S rRNA gene, V9	Dairy products: soft cheese, yoghurt, butter and bifido-drink	Kaufmann et al. (1997)
FISH	/	/	16S rRNA gene	Fermented food	Lahtinen et al. (2005)

Table 1 (*continued*)

Technique	Primer	Primer sequence 5′–3′	Gene	Application matrix in this review	Reference
Real-time PCR	dot AF	ATTGTCTCGCGCGATTGC	dotA gene	Water samples	Yáñez et al. (2005)
	dot AR	CCGGATCATTATTAACCATCACC			
Real-time PCR		AAGCCTTGCAGGACATCTTCA GCCGCCAGTGTGATGGATAT	Enterotoxin B	Cooked ham, tuna, paella, milk from milk powder and a mixture of caramel and coffee creamer	Rajkovic et al. (2006)
Real-time PCR	sakF	GATAAGCGTGAGGTCGATGGTT	IGS	Meat and fermented sausages	Martin et al. (2006)
	sakR	GAGCTAATCCCCCATAATGAAACTAT			
Real-time PCR	La1	GATCGCATGATCAGCTTATA	16S rRNA gene	Commercial fermented milk	Furet et al. (2004)
	La2	AGTCTCTCAACTCGGCTATG			
Real-time PCR	Lj1	CACTAGACGCATGTCTAGAG	16S rRNA gene	Commercial fermented milk	Furet et al. (2004)
	La2	AGTCTCTCAACTCGGCTATG			
Real-time PCR	Ld1	ACATGAATCGCATGATTCAAG	16S rRNA gene	Commercial fermented milk	Furet et al. (2004)
	Ld2	AACTCGGCTACGCATCATTG			
Real-time PCR	Lc3	GCGGACGGGTGAGTAACACG	16S rRNA gene	Commercial fermented milk	Furet et al. (2004)
	Lc4	GCTTACGCCATCTTTCAGCCAA			
Real-time PCR	Lc1	GTGCTTGCACTGAGATTCGACTTA	16S rRNA gene	Commercial fermented milk	Furet et al. (2004)
	Lc2	TGCGGTTCTTGGATCTATGCG			
Real-time PCR	Lp1	GTGCTTGCACCGAGATTCAACATG	16S rRNA gene	Commercial fermented milk	Furet et al. (2004)
	Lc2	TGCGGTTCTTGGATCTATGCG			
Real-time PCR	Lr1	GTGCTTGCATCTTGATTTAATTTT	16S rRNA gene	Commercial fermented milk	Furet et al. (2004)
	Lc2	TGCGGTTCTTGGATCTATGCG			
Real-time PCR	St1	TTATTTGAAAGGGGCAATTGCT	16S rRNA gene	Commercial fermented milk	Furet et al. (2004)
	St2	GTGAACTTTCCACTCTCACAC			
Real-time PCR	tdhF	TCCCTTTTCCTGCCCCC	Thermostable direct hemolysin	Oysters	Nordstrom et al. (2007)
	tdhR	CGCTGCCATTGTATAGTCTTTATC	Pathogenicity marker for *V. parahaemolyticus*		
Real-time PCR	trhF	TTGCTTTCAGTTTGCTATTGGCT	Thermostable-related hemolysin	Oysters	Nordstrom et al. (2007)
	trfR	TGTTTACCGTCATATAGGCGCTT	Pathogenicity marker for *V. parahaemolyticus*		
Real-time PCR	tlhF	ACTCAACACAAGAAGAGATCGACAA	Thermolabile hemolysin	Oysters	Nordstrom et al. (2007)
	tlhR	GATGAGCGGTTGATGTCCAA	Species-specific for *V. parahaemolyticus*		
SSCP	F w49	ACGGTCCAGACTCCTACGGG	16S rRNA gene, V3	Cheese, raw milk	Duthoit et al. (2003), Delbès et al. (2007)
	R w34	TTACCGCGGCGTGCTGGCAC			
SSCP	F V2F	GGCGAACGGGTGAGTAA	16S rRNA gene, V2	Cheese	Duthoit et al. (2003)
	R V2R	ACTGCTGCCTCCCGTAG			
T-RFLP	F 27F	[6FAM]AGAGTTTGATCMTGGCTCAG	16S rRNA gene	Cheese, yoghurt	Rademaker et al. (2005, 2006)
	R SD-BACT-0926	CCGTCAATTCCTTTRAGTTT			
T-RFLP	F 27F	[6FAM]AGAGTTTGATCMTGGCTCAG	16S rRNA gene	Sugar thick juice	Justé et al. (2008b)
	R 1387R	GGGCGGWGTGTACAAGGC			
T-RFLP	F 63F	[6FAM]CAGGCCTAACACATGCAAGTC	16S rRNA gene	Milk	Sanchez et al. (2006)
	R 358R	GCTGCCTCCCGTAGGAGT			
T-RFLP	F 63F	[6FAM]CAGGCCTAACACATGCAAGTC	16S rRNA gene	Atlantic cod (*Gadus Morhua*)	Wilson et al. (2008)
	R 1389R	ACGGGCGGTGTGTACAAG			

[a] Analyses were performed on isolates instead of whole communities.

be excised from the gel and identified by sequencing. However, reliable identification by sequencing may be hampered by the small fragment sizes of the PCR products, which might not contain enough information for precise taxonomic classification (Øvreås, 2000). In addition, it should be noted that different sequences may have identical electrophoretic mobility, resulting in co-migration of different fragments (Sekiguchi et al., 2001). A major drawback often associated with DGGE/TGGE is lack of reproducibility (Powell et al., 2005), as handling of big gels, primer–dimer formation and variable gel staining all affect reproducibility. However, reproducibility can be enhanced by the inclusion of an internal standard, facilitating normalization of samples within and between gels (Neufeld and Mohn, 2005; Petersen and Dahllöf, 2005). Another drawback often associated with these methods is low sensitivity due to traditional gel staining, resulting in the loss of bands representing less abundant community members. Nevertheless, the use of fluorescently labeled primers can improve the sensitivity of detection (Moeseneder et al.,1999; Neufeld and Mohn, 2005).

The first application of DGGE in food microbiology dates from 1999, when Ampe et al. reported on the spatial distribution of microorganisms in pozol balls, a Mexican fermented maize dough. Since then, several food or associated products have been analyzed with DGGE (Ercolini, 2004), including mineral water (Dewettinck et al., 2001), wine (Lopez et al., 2003), sausages (Cocolin et al., 2004; Rantsiou et al., 2005), dairy products (Ercolini et al., 2004; Lafarge et al., 2004; Ogier et al., 2004; Florez and Mayo, 2006), fermenting cassava dough (Miambi et al., 2003), beef (Ercolini et al., 2006), fish (Giacomazzi et al., 2004; Hovda et al., 2007a, b).

3.1.2. Single-strand conformation polymorphism

Another technique that relies on electrophoretic separation of PCR products and that has been used for the analysis of microbial communities is SSCP (Orita et al., 1989; Lee et al., 1996). Like DGGE and TGGE, this method allows separation of different DNA fragments of similar length. In contrast to DGGE/TGGE, SSCP separates PCR products based on conformational differences of folded single-stranded products. Following denaturation, single-stranded DNA fragments are loaded on a non-denaturing acrylamide gel. Under non-denaturing conditions a stable secondary structure is formed which is mainly determined by the intramolecular interactions that depend on the nucleotide sequence. Based on the migration of these secondary structures in the gel, products with similar molecular weight can be separated and visualized. In general, SSCP has the same advantages and limitations as DGGE or TGGE. However, since no clamped primers are required, PCR amplification is more robust than with DGGE or TGGE. However, a major limitation of SSCP is the formation of several stable conformations out of one single-stranded DNA fragment, resulting in multiple bands on gel (Tiedje et al., 1999). Furthermore, a high rate of re-annealing of DNA strands during electrophoresis has been reported (Selvakumar et al., 1997), especially in the analysis of high-diversity communities since these samples typically require high DNA concentrations for loading (Schwieger and Tebbe, 1998). However, this problem can be circumvented by using a 5′-phosphorylated primer (Schwieger and Tebbe, 1998) which allows selective removal of the corresponding phosphorylated strand through digestion with lambda exonuclease.

SSCP has been applied to study microbial communities in different matrices ranging from water (Lee et al., 1996; Lin et al., 2007) and soils (Sliwinski and Goodman, 2004; Witzig et al., 2006) to anaerobic digestors (Zumstein et al., 2000; Leclerc et al., 2001). However, so far applications in food microbiology have been limited to cheese (Duthoit et al., 2003; Delbès et al., 2007). In addition to the analysis of microbial communities, this technique has been used widely in mutation analysis (Hamzeiy et al., 2002) as well as for the differentiation and typing of isolates (Wagner et al., 2000; Moore et al., 2000).

3.1.3. Terminal restriction fragment length polymorphism

T-RFLP analysis is another PCR-based community profiling method that is commonly used for comparative microbial community analysis. Marker genes are amplified with (a) fluorescently labeled primer(s), followed by restriction digestion (typically using 4-base cutters) and separation and detection on an automated sequencer (Liu et al., 1997). Only labeled terminal restriction fragments (TRFs) are detected and their length heterogeneity indicates the complexity of the community visualized by an electropherogram. An internal size standard, labeled with a different fluorescent dye, allows precise length assignment with single-base pair resolution.

With the 16S rRNA gene as target, obtained TRFs can be compared to the rapidly expanding sequence database of the Ribosomal Database Project (RDP; Cole et al., 2005), allowing predictions of the organisms present in the analyzed sample (Marsh et al., 2000). Because one restriction enzyme often does not provide sufficient resolution (Marsh, 2005), multiple restriction enzymes are typically used, increasing the specificity and the reliability of the assay (Osborne et al., 2006). The choice of the endonucleases is based on the desired degree of discriminative power, or to track a specific population. Web-based tools including the RDP (http://rdp.cme.msu.edu) and the Microbial Community Analysis (MiCA) website (http://hermes.campus.uidaho.edu) allow the *in silico* prediction of TRFs, enabling selection of endonucleases.

T-RFLP allows very sensitive detection and because of its high-throughput capacity, it performs well in surveys with large sample numbers, e.g. to ascertain spatial or temporal changes in a community structure. However, this method also has some limitations to accurately predict microbial community structures. Incomplete or non-specific restriction leads to overestimation of the diversity since the number of fragments increases. However, restriction efficiency can be checked, e.g. by inclusion of an internal standard in the restriction step (Marsh et al., 2000). Overestimation of diversity can also be generated by pseudo-terminal restriction fragments as reported by Egert and Friedrich (2003). These fragments can be produced upon intramolecular folding of single-stranded products, creating transient structures that are accessible for digestion. However, this problem can be addressed by treatments with single-strand-specific nucleases (Egert and Friedrich, 2003). Apart from these limitations related to the determination of the diversity within a community, one of the major restrictions for identification is variation between the *in silico* predicted and the experimentally obtained TRF lengths (Kaplan and Kitts, 2003; Pandey et al., 2007). Kaplan and Kitts (2003), for example, measured mobility differences within phylogenetic groups, presumably due to sequence variability, and also detected striking mobility variations caused by fluctuations in ambient temperature between runs. As a consequence, tolerance ranges for length assignment from 1 to 2 bases are generally used to allow matching with database entries, which, in turn, increase the number of species associated with a TRF. Another sizing variable is introduced by the choice of the fluorescent dye (Pandey et al., 2007). Therefore, the primers and labeling dyes should be carefully evaluated, optimized and standardized for an individual community under investigation.

Despite these limitations, T-RFLP has become a valuable molecular tool for microbial community analysis, especially when high throughput and high sensitivity are required without the need for direct sequence information. The usefulness of T-RFLP in microbial ecology has been extensively demonstrated in diverse research domains (Jernberg et al., 2005; Rasche et al., 2006). Increasingly, also in the food industry, T-RFLP is successfully used to describe microbial populations, e.g. in fishery (Wilson et al., 2008), yogurt and cheese production (Rademaker et al., 2005, 2006; Sanchez et al., 2006) and the sugar industry (Justé et al., 2008b).

3.1.4. Amplified ribosomal DNA restriction analysis

Amplified ribosomal DNA restriction analysis (ARDRA), also known as restriction fragment length polymorphism (RFLP) analysis of the 16S rRNA gene, is a relatively simple PCR-based fingerprinting technique based on the digestion of amplified ribosomal community DNA followed by gel electrophoresis that can be used for microbial identification (Laguerre et al., 1994) or comparison of microbial communities and dynamics (Moyer et al., 1994). In contrast to T-RFLP, all digested fragments are detected, increasing the level of resolution. Nevertheless, likewise the complexity of the profiles also increases, thus hampering comparison and interpretation of complex patterns. However, one single restriction enzyme generally does not provide sufficient resolution, and multiple restriction enzymes have to be used either separately or in combination to obtain the desired resolution. Another drawback of this method is the limited staining sensitivity in gels resulting in the suppression of bands from less abundant community members or in a loss of phylogenetic information (Tiedje et al., 1999). As a consequence, this technique is only preferred when the community is

dominated by a few members (Fries et al., 1997), but is of limited use for demonstrating the presence of specific phylogenetic groups or for estimating complexity of communities. Acinas et al. (1997) reported the use of ARDRA fingerprinting to study spatial and temporal variation in bacterial marine plankton diversity. Nevertheless, despite its potential, to our knowledge, this method has not yet been used to study microorganisms in food.

3.1.5. (Automated) ribosomal intergenic spacer analysis

Ribosomal intergenic spacer analysis (RISA) (Borneman and Triplett, 1997) involves PCR amplification of total bacterial community DNA of the intergenic region between the 16S and 23S ribosomal genes. This intergenic spacer (IGS) region displays significantly more heterogeneity in length and nucleotide sequence than the flanking 16S and 23S ribosomal genes. In RISA, size differences of the spacers are exploited for subtyping of bacterial strains or in cases where fingerprinting of ribosomal sequences does not provide sufficient resolution (Kirk et al., 2004; Scheinert et al., 1996). In general, intergenic spacer lengths may vary between approximately 400 and 1200 base pairs (Fisher and Triplett, 1999). After gel electrophoresis of the PCR product, a complex community-specific banding pattern is generated, with each band corresponding to at least one organism in the original community. The lack of sensitivity associated with this gel-based method led to the development of automated RISA (ARISA; Fisher and Triplett, 1999), in which the original steps of DNA extraction and PCR amplifications are identical, except that a fluorescently labeled primer is used in the PCR. The electrophoretic step is subsequently performed on an automated system, with laser detection of fluorescent DNA fragments.

Potential problems associated with (A)RISA are the preferential amplification of shorter templates (Fisher and Triplett, 1999) and the fact that, because of IGS length variation within a single genome (Mateos and Markow, 2005), a single organism can contribute to more than one signal. In order to increase reproducibility and standardization, Cardinale et al. (2004) evaluated different primer sets with respect to universality, sensitivity and reproducibility and selected the most suitable primers for ARISA of environmental bacterial communities.

As for DGGE/TGGE and T-RFLP, ARISA has been regularly applied in microbial ecology (Ranjard et al., 2001; Schloss et al., 2003; Brown et al., 2005). However, only one study (Cardinale et al., 2004) reports on a community analysis of food samples, i.e. goat milk and ensiled corn. Nevertheless, for the molecular characterization of food isolates, ARISA has been frequently exploited (Kabadjova et al., 2002; De Angelis et al., 2007).

3.2. Identification

For many processes and matrices in which microorganisms are present, it is important to study not only the microbial diversity and differences between different samples, but also to precisely identify the key microorganisms. Some examples include the search for microorganisms that discriminate a disease suppressive from a disease conducive soil (Hunter et al., 2006), for microorganisms which hamper the production process of food (Justé et al., 2008a, b) and for bacteria that are responsible for the characteristic flavor and taste of certain foods such as cheese (Duthoit et al., 2003).

The body of DNA sequences from phylogenetic studies in public databases (Olsen et al., 1992; Benson et al., 2004; Cole et al., 2005; D'Auria et al., 2006) facilitates rapid identification of community members at the taxon level by sequencing and searching the databases for significant sequence homology. In addition, rapid developments in sequencing and DNA handling techniques have made it possible to routinely sequence partial-gene or whole-gene amplicons as an identification technique. However, new technologies such as DNA arrays provide even more attractive, high-throughput platforms that allow affordable identification of multiple organisms (or genes) based on specific, short DNA segments (Lievens et al., 2005b; Summerbell et al., 2005). The usefulness of both techniques in molecular ecology is discussed below.

3.2.1. Analysis of clone libraries

In order to identify (differential) signals obtained with the common community profiling techniques for gel-based approaches such as DGGE, TGGE, SSCP, ARDRA and RISA, bands can be excised from gels, cloned and sequenced (Lafarge et al., 2004). Alternatively, the PCR-amplified sequences can be directly cloned and sequenced, allowing species identification of individual community members. Increasingly, different matrices of which also foods, are analyzed by sequence analysis of 16S rRNA gene-based clone libraries. (Escalante et al., 2001; Singleton et al., 2001; Kim and Chun, 2005; Timke et al., 2005a, b). A major drawback of the use of clone libraries, however, is that, depending on the matrix, sometimes as many as a few thousand clones must be analyzed in order to cover the phylogenetic richness hidden in a prokaryotic gene library (Loy et al., 2006). As a result, wide application of gene library surveys has generally been limited in the past (Borneman and Triplett, 1997; Dunbar et al., 1999), irrespective of the continuous advances in high-throughput sequencing, rendering the application of clone libraries easier and more cost-effective. After all, this approach still remains tedious and time-consuming due to the large number of samples that has to be analyzed. Therefore, clone libraries are generally constructed in parallel to fingerprinting techniques like DGGE, T-RFLP or SSCP, that describe the complexity of the community and allow well-informed decisions on the number of clones to be sequenced (Duthoit et al., 2003; Handschur et al., 2005; Delbès et al., 2007; Justé et al., 2008b). Alternatively, restriction digestion of the clones may be helpful for initial screening of the clone library and distinguishing different restriction types which then can be sequenced (Lagacé et al., 2004; Kim and Chun, 2005).

Conventional DNA sequencing relies on the elegant principle of the dideoxy chain termination technique developed three decades ago (Sanger et al., 1977). However, this technique faces limitations in both throughput and cost. One of the most promising alternatives is called 'pyrosequencing' which allows high-throughput, and relatively cheap, sequencing (Ronaghi et al., 1996, 1998; Ronaghi, 2001). In addition, pyrosequencing eliminates the need for cloning, thus removing the potential for both production of aberrant recombinants and cloning-related artifacts (Speksnijder et al., 2001). Currently, pyrosequencing has been used in microbial community analyses of soils (Roesch et al., 2007) and mines (Edwards et al., 2006) and for the detection of single-nucleotide polymorphisms (Fakhrai-Rad et al., 2002). In order to expand the applications for pyrosequencing in microbial ecology, however, the read length, which is now limited to 200 base pairs, should be improved (Ahmadian et al., 2006).

An alternative to sequencing of the standard conserved genes is sequencing of randomly cloned community DNA, known as 'whole-genome shotgun sequencing' (Fleischmann et al., 1995). With this technique, genomic DNA is physically sheared and fractionated, followed by cloning and sequencing of the fragments. Multiple overlapping 'reads' for the target DNA are obtained by performing several rounds of this fragmentation and sequencing. Unlike the previous approaches, which typically study a single gene, this approach offers a more global view of the

community (Tyson et al., 2004; Venter et al., 2004; Tringe et al., 2005). In addition, whole-genome shotgun sequencing allows studying gene complements and metabolic pathways in a community, and in some cases, reconstruction of near-complete genome sequences. Furthermore, this approach has the potential to discover new genes that are too diverged from currently known genes to be discovered with PCR (Riesenfeld et al., 2004).

3.2.2. DNA array technology

In recent years, an increasing number of techniques has been deployed that use distinctive short nucleic acid segments (oligonucleotides) in identification procedures. Among these is DNA array technology, which was originally designed for gene expression or single-nucleotide polymorphism profiling (Saiki et al., 1989), the most suitable technique for identification of an, in principle, unlimited amount of unknown DNA sequences in a single assay (Lievens et al., 2005b). With this technique, specific detector oligonucleotides that are immobilized on a solid support hybridize with homologous-labeled target amplicons, which then can be detected. This strategy has proven to be successful for microbial identification, even when species can only be discriminated by a single-nucleotide polymorphism (Lievens et al., 2006b). With regard to the characterization of microbial communities, DNA arrays have the capacity to overcome some limitations associated with the restricted resolution of many fingerprinting methods, and the requirement of large sample numbers and associated costs for sequencing clones. In addition, since hybridization signals are proportional to the quantity of target DNA, this technique may also provide quantitative information (Cho and Tiedje, 2002; Lievens et al., 2005a).

Currently, two types of arrays have been developed, including low-density macroarrays (e.g. on a nylon membrane) and high-density microarrays (e.g. on a glass slide) which may contain up to hundreds or millions of detector oligonucleotides, respectively (Tambong et al., 2006; Xing et al., 2007). In general, DNA arrays can be divided into at least three categories based on the genes targeted by the array (Gentry et al., 2006). The first category encompasses the most common DNA arrays, so-called phylogenetic arrays, which are based on a diagnostic marker such as the 16S rRNA gene and are used for microbial identification. A second category comprises functional gene arrays, designed for the detection of key functional genes in a given environment. The third category consists of metagenomic arrays which, unlike the other arrays, contain DNA fragments produced directly from environmental DNA and can be applied with no prior sequence knowledge.

With regard to food microbiology, DNA array technology has successfully been used for direct description of microbial communities in MAP vegetable salads (Rudi et al., 2002) and for monitoring microbial populations during sugar thick juice storage (Justé et al., 2008c). In addition, DNA arrays have been widely used for the detection and identification of pathogenic and environmental microorganisms from various sources, including food, soil, animals and agricultural crops (Wilson et al., 2002; Lievens et al., 2003; Bodrossy and Sessitsch, 2004; Lievens and Thomma, 2005; Kostrzynska and Bachand, 2006; Eom et al., 2007). A major advantage of phylogenetic or functional gene arrays is the unlimiting expanding capacity to detect more and other microorganisms or genes of interest. However, this also implies its most important drawback since there is a need for specification of the target organisms or genes. As a result, DNA arrays are unable to identify taxa for which no oligonucleotides are developed yet. Furthermore, it is not always possible to have perfect specificity for all detector oligonucleotides since they all need to hybridize under the same conditions. Nevertheless, this problem can be addressed by spotting multiple oligonucleotides for the same target.

3.2.3. Fluorescence in situ hybridization

Fluorescence *in situ* hybridization (FISH) is another technique applied for bacterial identification and combines the simplicity of microscopy observation and the specificity of DNA hybridization (DeLong et al., 1989). Typically, FISH is based on the hybridization of labeled DNA probes to taxon-specific regions of the bacterial ribosomes and can be detected by fluorescence microscopy or flow cytometry (Amann et al., 1990; Wallner et al., 1993). In general, the whole procedure can be completed in a few hours (Amann et al., 1995).

As understanding the ecology of complex microbial communities is enhanced by analyses in minimally disturbed samples, FISH is widely used in environmental microbiology (Maszenan et al., 2000; Aminov et al., 2006). FISH technology reveals the morphology of the target organisms, how abundant they are in a given environment and what other organisms they associate with (Madigan et al., 2000). In food microbiology, FISH has been used for several applications including detection and identification of lactic acid bacteria in wine (Sohier and Lonvaud-Funel, 1998) and on the surface of gruyere cheese (Kollöffel et al., 1999), as well as for enumeration of probiotic bifidobacteria in fermented food (Kaufmann et al., 1997; Lahtinen et al., 2005) and carnobacteria and *Lactobacillus brevis* from sea food (Connil et al., 1998).

A recent advance in FISH technology is the replacement of standard linear oligonucleotide probes by molecular beacons resulting in better discriminatory power (Xi et al., 2003; Lenaerts et al., 2007). In addition, the use of molecular beacons reduces the need for washing, resulting in a reduced cell clumping and cell loss (Sekar et al., 2004) and a more accurate enumeration (Wallner et al., 1993). Peptide nucleic acids (PNAs) have also been used to achieve better resolution (Prescott and Fricker, 1999; Xi et al., 2003), but low signal-to-noise-ratios, long hybridization times and high costs limit their use.

Although in theory, FISH could detect single cells, in practice, however, the detection level is often 10^3 cells per ml, rendering this technique in general less sensitive than PCR-based techniques (Hogardt et al., 2000; Moreno et al., 2003; Poppert et al., 2005). Another limitation is the insufficient automation for high sample throughput (Amann et al., 2001). Furthermore, an extensive knowledge of the community is required since the probes need to be designed beforehand and after all, only a limited number of probes can be applied in one hybridization run. This last limitation becomes a distinct bottleneck when FISH is used for community analysis on a high level of phylogenetic resolution.

3.3. Quantification

Apart from knowing the identity of the (dominant) members of a community, in many cases it is also desirable to know the size of the respective populations, e.g. if its magnitude is expected to be pernicious in the case of pathogens, or beneficial in the case of probiotics. Therefore, in addition to describing the microbial community structure and identity determinations, quantification of the microbial populations is becoming more and more significant.

In order to quantify the presence of certain microorganisms by DNA-based techniques, the amount of genomic DNA should be correlated to the amount of biomass. However, the non-linear nature of PCR-amplification makes it challenging to relate the final amount of sequences to the initial amount of genomic DNA in the sample. As a consequence, PCR-based fingerprinting techniques such as DGGE and T-RFLP or DNA arrays are often considered

to be semi-quantitative (Lievens et al., 2005a; Sanchez et al., 2006). In contrast, accurate quantification of DNA can be performed using real-time PCR (Heid et al., 1996). Compared to conventional PCR, this method enables an online detection of the PCR product, avoiding the need for post-PCR processing. In addition, real-time PCR allows accurate template quantification over a wide dynamic range ($>10^7$-fold) (Bustin et al., 2005). Typically, DNA amplification is continuously monitored based on the emission of fluorescence. In general, the initial concentration of target DNA is linked to a precise threshold cycle, defined as the cycle number at which fluorescence increases above the background level. Ultimately, the target DNA is quantified using a calibration curve that relates threshold cycles to exact concentrations of template DNA. As widely discussed in other reviews (Mackay, 2004; Lievens et al., 2005b), accumulating amplicons can be detected with several chemistries that make use of either fluorescent DNA-intercalating dyes or sequence-specific fluorescent probes. In food microbiology, real-time PCR has profoundly been used to detect and quantify specific target organisms (Furet et al., 2004; Yáñez et al., 2005; Rajkovic et al., 2006; Martin et al., 2006; Nordstrom et al., 2007). However, the total amount of PCR reactions in one single tube is currently restricted to a handful of targets, because of the limited number of different fluorescent dyes available and the nature of the energizing light source that can be used in real-time instruments (Mackay et al., 2002), limiting a wide use in quantitative community analyses of complex matrices. Therefore, one of the present challenges in molecular biology is the development of quantitative multiplex assays that can effectively screen large numbers of targets in a given sample (Lievens and Thomma, 2007).

3.4. Examples of applications

Some of the techniques described in this review are being implemented increasingly in different aspects of food microbiology. Certain detailed examples of applications are described in this section. Other examples, and especially those related to studying the microbial ecology of fermented foods can be found in the recent book review of Cocolin and Ercolini (2008).

3.4.1. Monitoring population dynamics in sugar thick juice during storage to prevent sugar losses

Sugar thick juice is one of the intermediate products in the production of beet sugar, more precisely the concentrated juice after evaporation with a total soluble solids content of about 69°Bx and a slightly alkaline pH of 9.0. Storing thick juice beyond extraction and refining is common practice in the sugar industry. However, even under good storage practices, thick juice degradation resulting from microbial contamination still occurs. The most pronounced symptoms of degradation are a reduction in pH from 9 to 5–6 and typically, an increase in reducing sugar content due to microbial growth (Sargent et al. 1997; Willems et al., 2003), resulting in financial loss. Improving the control of these microflora-related problems requires a greater understanding of the microbial dynamics of thick juice storage.

Until 2007, literature on this subject was scarce and microbiological analyses of thick juice only occurred with classical techniques. Nevertheless, one of the major limitations associated with these techniques is the time consuming nature since many different media and incubation conditions are necessary to represent the total (culturable) microflora (Willems et al., 2003). Recently, thick juice microflora has been profoundly investigated with molecular tools, encompassing the application of 16S rRNA gene clone libraries and T-RFLP analysis, providing a more comprehensive representation of the thick juice microflora than the previous studies (Justé et al., 2008b, d). In these studies it was shown that different bacterial populations occur right after production or after long-term storage. However, the initial, heterogeneous microflora after thick juice production evolved to the dominance (>99%) of *Tetragenococcus halophilus* during storage. In addition, other bacteria such as *Staphylococcus* and *Bacillus* species were consistently present in lower steady concentrations of 10^3 cfu/ml (Justé et al., 2008b). Based on its high population density (10^6–10^7 cfu/ml) and ability to consume sucrose, *T. halophilus* is believed to play a crucial role in thick juice degradation (Justé et al., 2008b). Nevertheless, although the other detected bacteria are present in much lower concentrations, they may be able to contribute to thick juice degradation as well. In order to be able to detect the different bacteria that may occur in thick juice and may cause thick juice degradation, a DNA array was developed containing detector oligonucleotides for the genera *Bacillus*, *Kocuria*, *Staphylococccus*, and *Tetragenococcus*, and the species *Aerococcus viridans, Leuconostoc mesenteroides* and T. *halophilus* (Justé et al., 2008c). The developed macroarray was considered reliable and sensitive (up to 0.1 pg target DNA) and has potential for monitoring the thick juice microflora during storage. Currently, DNA array analyses are routinely performed for sugar refineries in order to monitor the microbiological quality of thick juice. Depending on the organisms detected, the companies are advised for further stable storage or direct processing of the valuable thick juice instead of loosing sugar to microbial degradation.

3.4.2. Evaluation of modified atmosphere packaged (MAP) fish products for safe storage

Food quality depends directly on the nature of the product, as well as on the handling and storage conditions. Modified atmosphere packaging (MAP) of fresh foods, often in combination with low-temperature storage, has been proven to extend product shelf life by limiting microbial growth (Faber, 1991). In order to determine the best storage conditions, identification and characterization of the microorganisms which have the ability to grow under these limiting conditions are important. In addition, thorough characterization of the total microflora will increase our knowledge about potential hazards in controlled food production, when, for example, the hazard analysis of critical control point (HACCP) concept is used. However, thus far, the main focus has been on the detection of culturable, specific bacteria, mainly spoilage bacteria (Gram and Huss, 1996), while very little was known about the total microbial flora (Rudi et al., 2004). Nevertheless, whole community analysis techniques such as DGGE, T-RFLP or 16S rRNA gene clone libraries are used increasingly to characterize the predominant microbial flora during MAP storage from e.g. fresh fish (Jensen et al., 2004; Rudi et al., 2004; Hovda et al., 2007a). With these methods a more diverse bacterial flora was detected than previously obtained with culture-based methods. For example, Rudi et al. (2004) investigated the microbial diversity of MAP salmon and coalfish using a combination of molecular techniques, including 16S rRNA gene sequencing of pure cultures, real-time PCR, T-RFLP analysis and 16S rRNA gene clone libraries. Amongst multiple bacterial taxa, the authors found a strong association of coalfish with *Photobacterium phosphoreum* whereas *Brochothrix* and *Carnobacterium* species were associated with salmon. In comparison with classical plating techniques, several bacteria, including *Streptococcus* spp., propionibacteria and clostridia were only detected by the molecular techniques. Interestingly, by using direct cloning and sequencing, a clone with 100% 16S rRNA gene identity to *C. botulinum,* reported as an animal and human pathogen, was identified. Undoubtedly, the detection of such microorganisms

which could not be detected before will lead to more scientifically substantiated definitions for advisable storage practices.

3.4.3. Categorizing biofilms as a potential source of microbial contaminants

Biofilms consist of complex aggregations of microorganisms and are ubiquitous in nature. In addition, they appear to be more resistant to environmental stresses than their free-living counterparts (Costerton et al., 1999). Biofilms can display either beneficial or detrimental properties, depending on the environment and situation where they are formed. In food processing environments, biofilms are a major concern and may occur in areas where food is stored or on food processing surfaces such as conveyer belts and stainless steel (Kumar and Anand, 1998). In the meat industry for example, harmful, surviving microorganisms from carcasses (e.g. *Listeria* species) can contaminate surfaces where products are placed (Gandhi and Chikindas, 2007). In the brewing industry, beer-spoiling organisms such as members of the Lactobacillaceae (e.g., *Lactobacillus brevis* and *L. lindneri*) and the Acidaminococcaceae (e.g. *Pectinatus cerevisiiphilus*, *P. frisingensis*, and *Megasphaera cerevisiae*) can establish themselves in existing biofilms and potentially damage the end product (Timke et al., 2004). Consequently, the striking interrelations between product spoilers and biofilm-forming microorganisms emphasize the importance of the composition of the biofilm community. There were several attempts to characterize biofilms by culture-dependent techniques (Vaisanen et al., 1998). However, these approaches provided only an incomplete picture of the community. In contrast, Timke et al. (2005b) analyzed the microbial composition of biofilms from a beer bottling plant with 16S rRNA gene clone libraries to investigate their role in beer spoilage. Although an unexpected diverse microflora was identified, no important beer spoiling bacteria were detected in the biofilm. Nevertheless, by using a more extensive experimental set up, the authors were able to detect some beer-spoiling genera in a few samples (Timke et al., 2005a). Interestingly, the genus *Methylobacterium* which can be readily overlooked by cultivation approaches, was one of the dominating groups of the clone libraries (Timke et al., 2005b). In addition, considerable numbers of clones were assigned to non-culturable bacteria, illustrating the usefulness and power of culture-independent techniques to describe the whole microbial community and to categorize biofilms as a potential source of microbial contaminants.

4. General pitfalls and limitations

Molecular techniques have become increasingly important in the characterization of microbial communities in terms of community structure, identification and quantification. However, despite all their advantages there remain limitations to molecular techniques that must be considered when performing and interpreting community analyses.

4.1. Sampling

One of these potential bottlenecks are sampling procedure and sample size. As the amount of material necessary for analysis reduces with the development of more sensitive techniques, developing appropriate sampling strategies is becoming even more challenging. Assessing the microbial community in a given environment requires a well-thought sampling plan that ensures a statistically representative sample. In addition, to compare different analyses, a standard sampling procedure and sample size should be used. However, pooling multiple small samples into one extraction or using subsamples from a homogenized bulk sample may be the preferred sampling method. Different food sampling strategies were recently discussed by Holden (2007). After sampling, sample treatment is the next crucial step and the choice for aerobic or anaerobic storage, washing, freezing or refrigeration procedures may alter the original microbial community and may therefore well be considered.

4.2. DNA extraction and PCR amplification

Most community analysis studies are based on the extraction of total community DNA, followed by PCR amplification of the nucleotide sequence of interest. Nevertheless, it is known that this relatively straightforward approach may be interspersed with pitfalls.

Whether or not molecular techniques represent all microorganisms present in a sample depends first of all on the extraction efficiency of the nucleic acids of the different taxa in the sample. In addition, the presence of natural compounds such as polysaccharides, fat, carbohydrates, proteins or salts may hamper DNA extraction (Rossen et al., 1992; Wilson, 1997). Furthermore, these compounds may also affect PCR efficiency. As a result, these inhibiting substances need to be removed during DNA extraction or amplification enhancers should be added (Simon et al., 1996). Nevertheless, in many cases these problems can be directly circumvented by the use of commercially available extraction kits. However, the presence and effect of inhibitors in the DNA extract always needs to be tested with proper controls. Although the purity of the extracted nucleic acids is crucial for efficient PCR amplification and subsequent community analysis, bias may as well be introduced by the PCR and the selected primers. The choice of the primers is not always trivial since primers that are believed to be 'universal' do not always amplify all targets with the same efficiency and are thus not perfectly universal (Forney et al., 2004). Furthermore, many sequences in public databases are incomplete and even more sequences are unknown. As a consequence, whole groups might be excluded by the chosen primers (Marchesi et al., 1998; Wantabe et al., 2001; Osborn et al., 2000; Hongoh et al., 2003). Degenerate primers are used when highly conserved domains are lacking as annealing sites for universal primers (Nicolaisen and Ramsing, 2002). Nevertheless, an increasing degree of degeneracy increases the risk of aspecific DNA amplification and introduction of bias in the PCR reaction (Polz and Cavanaugh, 1998). In addition, PCR amplification may introduce an additional source of bias. Profiles generated by PCR-based methods are a reflection of the pool of PCR products, and do not necessarily represent the original microflora. Preferential amplification of abundant sequences (Chandler et al., 1997), different amplification efficiency (Suzuki and Giovanni, 1996; Polz and Cavanaugh, 1998), differences in gene copy number (Crosby and Criddle., 2003), and formation of chimeric sequences (Wang and Wang, 1997) all decrease the reliability of the presented biodiversity in microbial communities after PCR. In addition, in order to obtain an accurate representation of the microbial community, the number of PCR cycles needs to be optimized to ensure analysis of PCR fragments that were still in the exponential phase of the reaction without loosing information on minor populations (Suzuki and Giovanni, 1996). However, the problem of a non-detection of minor populations in the presence of (a) dominant population(s) may be addressed by several approaches. For example, Hancock et al. (2002) proposed 'PCR clamping' using PNAs (Ørum et al., 1993) to suppress PCR amplification of dominant sequences. PNAs recognize and bind to their complementary nucleic acid sequences with higher thermal stability and specificity than the corresponding

deoxyribooligonucleotides. Since these molecules cannot function as primers the PNA/DNA complex effectively blocks the formation of a PCR product when the PNA is targeted towards one of the PCR primer sites. Furthermore, PCR blockage can be accomplished when the PNA target sequence is located between the PCR primers. However, a disadvantage of this approach is that the initial amount of target sequences is influenced in an uncontrolled manner. More recently, Green and Minz (2005) described a suicide polymerase endonuclease restriction (SuPER) PCR to augment amplification of minor templates while dominant sequences are selectively inhibited from PCR. This SuPER PCR is based on a discriminating restriction of dominant sequences after pre-amplification with taxon-specific primers, making these sequences unavailable for subsequent amplification with universal primers and thus enhancing amplification of sequences from minor populations. Essential in this approach are designing a PCR to selectively amplify dominant sequences and the availability of a thermotolerant restriction enzyme with selective cutting. Another possibility to eliminate a dominant population is to screen for a restriction enzyme that only cuts the sequence of the dominant population. However, depending on the universality of the target sequence, such enzymes might not exist.

4.3. Viable versus non-viable

DNA is a highly attractive target for molecular studies because it is easy to handle and fairly resistant to degradation. In addition, with improved extraction methods and commercially available extraction kits, highly purified DNA can rather easily be obtained from complex environmental samples (McCartney et al., 2003; Lievens et al., 2005b). However, DNA-based techniques have sometimes been criticized because they do not distinguish living from non-living organisms (Rudi et al., 2005). In spite of that, it is generally accepted that DNA from dead cells will be metabolized quickly by other microorganisms in microbiologically active environments, such as humid or aquatic environments (Lebuhn et al., 2004). However, the rate of DNA degradation is very slow in matrices that are rich in easily degradable sugars like sugar thick juice. In this matrix, DNA from dead bacteria was found to be stable for over 40 days, overestimating certain populations in the community (Justé et al., unpublished results).

To exclude detection of non-viable organisms, DNA-based techniques may be combined with an enrichment step (Schaad et al., 1995). However, major disadvantages of this approach are the implication for quantification since the initial amount of target is influenced in an uncontrolled manner, and the inability to detect organisms that are either slow growing or non-culturable. Perhaps a more attractive alternative is the use of certain chemicals such as ethidium monoazide (EMA; Rudi et al., 2005) or propidium monoazide (PMA) to differentiate between viable and non-viable organisms. Both these chemicals can penetrate comprised membranes (that generally occur in dead cells), after which they bind to DNA after photo-induction of the azide group. This process renders the DNA insoluble and is therefore removed during DNA extraction. PMA only penetrates into dead cells, whereas EMA is proven to incorporate in living cells as well, leading to substantial loss of DNA (Nocker et al., 2006). Alternatively, RNA can be used as a target instead of DNA, in combination with reverse transcriptase PCR (RT-PCR; Tan and Weis, 1992). Since RNA is less stable than DNA, RNA will be degraded more quickly in dead organisms. In addition, it is believed that RNA-based assays are more sensitive than DNA-based assays. This was demonstrated in a recent medical study in which more patients were found to be infected with *Chlamydia trachomatis* through an rRNA test than through a DNA-based test (Yang et al., 2007). This may be explained by the fact that bacterial rRNA is present in amounts up to 10,000 times that of genomic DNA. However, because of its extreme sensitivity to degradation, specific precautions should be taken to extract RNA from environmental samples (Davis et al., 2006).

4.4. Active versus non-active

Another fundamental issue in microbial ecology concerns the relationship between microbial diversity and the biogeochemical function in a certain ecosystem. While most of the currently applied analysis methods, starting from environmental DNA, deliver information about the microbial history, presence or diversity, they provide no information about their level of physiological activity. In contrast, messenger RNA (mRNA) is only produced by metabolically active cells and therefore selectively detects active microbial populations. Moreover, additional information on the functioning of the microbial community is provided (Bodrossy et al., 2006).

4.5. Sequence databases: availability and quality

Phylogenetic and genome studies are showing unequivocally that some parts of the genome are highly conserved across large taxonomic groups, whereas others are hypervariable. Because of this fundamental nature of genomes and the rapidly expanding DNA sequence databases, there is a huge amount of data to identify, or at least compare, unknown sequences at different taxonomic levels. Nevertheless, a significant proportion of the organisms represented in these databases is historically misidentified, and many more are unnamed (Crous, 2002). In addition, the quality of some sequences is not always optimal or sufficient, indicating care should be taken when interrogating these databases.

4.6. Automation

Currently, there is a clear trend towards automation of community profiling, increasing reproducibility and minimizing handling and costs. In many cases, automation results from the use of capillary technology instead of acrylamide gels, which, in addition to automation, also increases the detection limit of the analysis. For example, when both DGGE and T-RFLP were used to detect different ribotypes in various soil samples, T-RFLP was found to be at least five times more sensitive (Tiedje et al., 1999). Nevertheless, currently, automated sequencers are still very expensive. In addition, analysis of fluorescently labeled PCR products may be perverted by the fluorophores, as there may be differences in the expected and observed length of the products (Kaplan and Kitts, 2003; Pandey et al., 2007).

4.7. Data analysis and interpretation

The ultimate goal of ecological studies is to understand dynamic processes and diversity in microbial communities. In general, the obtained amount of microbial data regarding communities, added to other relevant ecological data such as those obtained by chemical or physical analyses, is often phenomenal and therefore a major statistical challenge. Most obstacles encountered by microbial ecologists when they try to summarize and further explore large data sets concern the choice of the adequate numerical tools to further evaluate the data statistically and visually. Increasingly, specific software packages are being developed substantially improving the way in which these datasets can be analyzed, including T-RFLP data

(Marsh et al., 2000; Kent et al., 2003; Shyu et al., 2007), analysis of gels (e.g. Gelcompar), DNA array data (Allison et al., 2006), or genomics and proteomics in general (Zeeberg et al., 2003; Glasner et al., 2006). The ultimate goal is to understand and model the main dynamic interactions in complete microbial communities. Nevertheless, skilled statisticians are scarce and every software has its own advantages and limitations, making straightforward analysis and interpretations still the Achilles heel of community analysis. Detailed reviews are provided by e.g. Rudi et al. (2007), Rudi (2008) and Ramette (2007) but are not under discussion in this review.

5. Concluding remarks

With the advent of PCR and its many derived techniques, the field of microbial ecology has evolved with increasing speed over the past two decades. Moreover, now that a number of molecular techniques has been established in microbial ecology, the next challenge is to properly analyze and interpret the obtained data in relation to different other factors (Allison et al., 2006; Ramette, 2007). In food microbiology, however, the trend of molecular ecological studies is only getting started. In general, the choice of an appropriate technique to study microbial communities depends on the aims of the research, the complexity of the community, the expertise of the lab personnel, and the required resolution and sensitivity level. However, when it comes to routinely monitoring a certain ecosystem on pre-defined characteristics, criteria such as cost of the analysis and high-throughput sample analysis are additional important requirements. Fingerprinting techniques such as T-RFLP, TGGE/DGGE and SSCP produce a rough view on the microbial community composition and provide relevant data for subsequent in-depth analysis. Indeed, in combination with sequencing or clone library analysis, a more detailed profile can be obtained, allowing the design of DNA arrays and/or real-time PCR assays as presented in

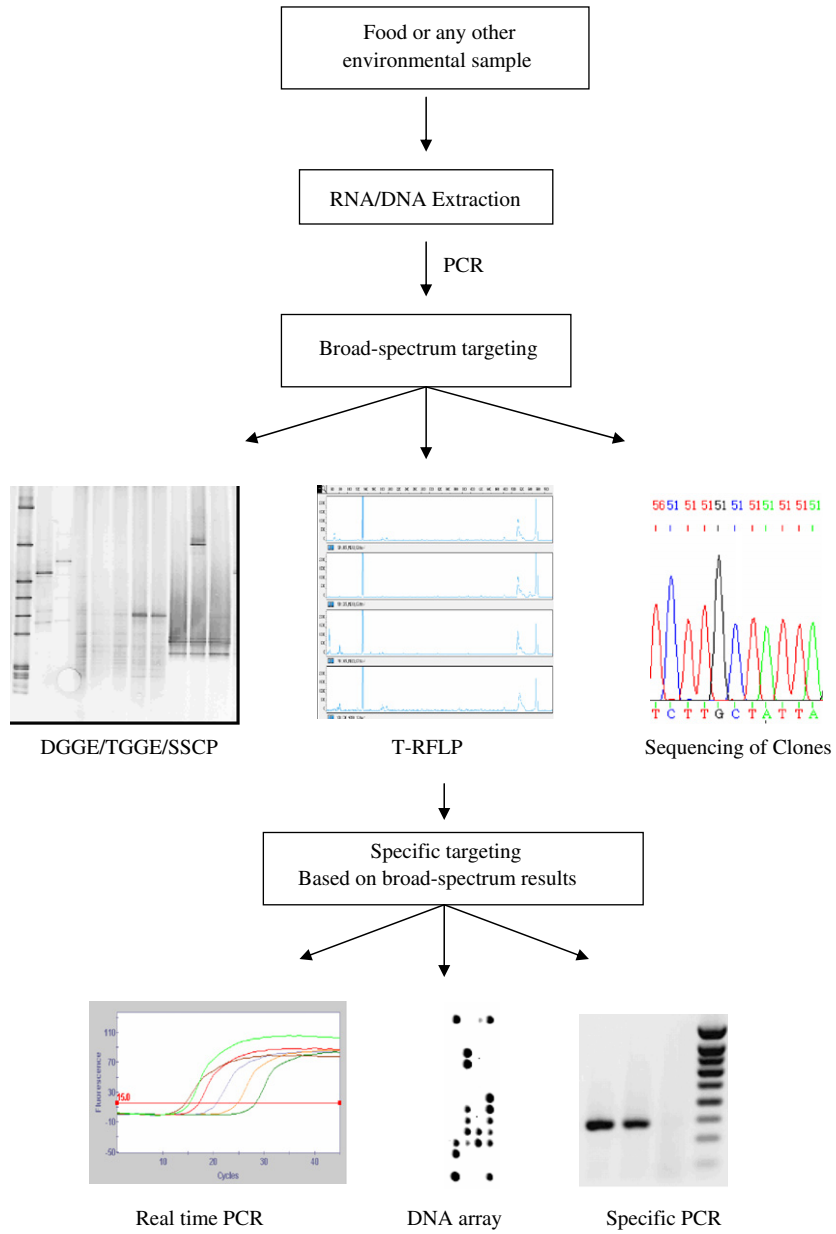

Fig. 1. Flow diagram of potential development of a monitoring tool for microbial community analysis in food samples. Starting from extracted nucleic acids, a whole community approach produces an overview of the total microflora, after which a more specific and detailed monitoring tool can be developed based on the obtained results.

Fig. 1. Dominant species or any species of interest can be detected reliably and quantified with a phylogenetic DNA array. In addition, taxonomically different species that share the same function can be characterized by a functional array. Alternatively, real-time PCR assays can be developed to assess these targets specifically. Advantageously, such straightforward assays can be performed by diagnostic laboratories (Lievens and Thomma, 2005), enabling routine screening of food quality and safety. Moreover, the strongly recommended implementation of HACCP (Zhao et al., 2001; Kokkinakis and Fragkiadakis, 2007) is enhanced. As soon as such molecular tools become available and affordable for the food industry, they will probably be implemented into food quality and safety control programs. One example is the implementation of PCR-based pathogen detection assays which has now become routine in food analysis, compared to its sporadic use ten years ago.

Nevertheless, the use of molecular techniques does not have to exclude traditional microbial techniques. On the contrary, they can be used together to acquire more accurate and comprehensive results. Moreover, as our knowledge of microbial diversity increases and availability of large genomic datasets grows, the role of cultivation in environmental microbiology may enlarge again (Tyson and Banfield, 2005). Genetic and metabolic information may circumvent the bottlenecks that have hindered cultivation of many microorganisms, providing a more comprehensive picture of the total community. Indeed, it already appears now that for at least some of the so-called 'non-culturable organisms', it is the inability to compete on nutrient-rich media which makes them hard to isolate and grow. Dilution to extinction (Wise et al., 1999) and low nutrient media (Aagot et al., 2001) can help to culture some of these organisms, an important step to better characterize unknown species.

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