



Culture independent methods to assess the diversity and dynamics of microbiota during food fermentation

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ARTICLE INFO

Available online 20 May 2013

Keywords:

Culture independent methods
DGGE
Food fermentation
Food spoilage
Microbial ecology

ABSTRACT

Culture independent methods first appeared in the food microbiology field at the end of the 90s and since then they have been applied extensively. These methods do not rely on cultivation and target nucleic acids (DNA and RNA) to identify and follow the changes that occur in the main populations present in a specific ecosystem. The method that has most often been used as a culture independent method in food microbiology is denaturing gradient gel electrophoresis (DGGE). The number of papers dealing with DGGE grew exponentially in the late nineties and, by analysing the studies available in the literature, it is possible to describe a trend in the subjects that have been investigated. DGGE was first used as a tool to monitor the ecology of fermented food, such as fermented sausage, cheese and sourdough, and later it also showed its potential in microbial spoilage process. In the last few years, the main application of DGGE has been to study fermented food from Asia, Africa and South America.

The information collected using DGGE has made it possible to confirm the existing knowledge on food fermentation and spoilage. However, in some cases, new evidence that helps scientists to fully comprehend a specific microbial ecosystem has emerged.

In this review, the roadmap of culture independent methods in food microbiology will be summarized, focusing on the DGGE technique. Examples of how this approach is useful to obtain a better understanding of microbial diversity are reported for several kinds of fermented food, such as fermented sausage, cheese and wine.

The future of culture independent methods in food microbiology, with the increasing availability of next generation sequencing techniques, is also discussed.

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1. Introduction

The last 30 years have been characterized by a significant change in the approaches used for the microbiological examination of food. The invention of PCR (Mullis et al., 1986) has led to new strategies to study food-borne microorganisms. Although, in the past, synthetic media, which were used to cultivate microorganisms, were the only way of conducting a microbiological analysis of food, with the arrival of PCR it has become possible to investigate microorganisms without any cultivation. In its early stages in food microbiology, PCR was mainly used as a detection method (Rossen et al., 1991). At the end of the 90s, a number of techniques were developed and, coupled with PCR, these techniques offered scientists the possibility of studying the ecology of complex microbial ecosystems. In this context, the term “culture-independent techniques” was coined. This term indicates the use of methods that are not based on cultivation to study microorganisms in a specific ecosystem. Undoubtedly, culture-independent methods offer a number of advantages over culture-dependent methods. Microorganisms are studied not because they are able to grow on a specific microbiological

medium, but because they possess DNA, RNA and proteins, which are the preferred targets for such approaches. Moreover, the physiological status of the microbial cell does not affect the outcome of the investigation. In traditional microbiological examinations, cells that are stressed and injured are often not able to grow on synthetic media that contain agents, such as antibiotics, to make them selective towards a specific microorganism, and this can lead to false-negative results. Finally, populations that are numerically less important are not detected by means of traditional methods, because they are masked on the plates. Most of these issues can be solved by culture-independent methods (Cocolin and Ercolini, 2008).

The introduction of culture-independent methods allowed scientists to understand the limitation of microbial cultivation and, in 1998, Hugenholtz et al. published a paper in which it was stated that “our knowledge of the extent and character of microbial diversity has been limited, however, by reliance on the study of cultivated microorganisms. It is estimated that >99% of microorganisms observable in nature typically are not cultivated by using standard techniques”. Such evidence encouraged researchers to use culture independent methods in different fields of microbiology. In food microbiology, the late 90s–early 20s represents a key period for the application of these approaches and the first papers, dealing with

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the study of microbial ecology of fermented foods, started to appear in scientific journals. One of the aspects that was immediately highlighted by these pioneer studies was the presence of non culturable populations in food systems, and these outcomes were in agreement with results obtained in other branches of microbiology, such as environmental and intestinal microbiology. Since they had never been detected by traditional methods, these populations were described for the first time by culture independent methods. This state, defined as viable but not culturable (VBNC), was defined by Oliver (1993) as “a cell which is metabolically active, while being incapable of undergoing the cellular division required for growth in or on a medium normally supporting growth of that cell”, can be considered as a survival strategy and response to adverse environmental conditions (e.g. starvation or acid stress) (Rowan, 2004) that can easily be found in food fermentation and processing. The VBNC state is of concern when associated to food-borne pathogens, because there is a general lack of knowledge on the risks from VBNC cells. It cannot be assumed that such cells will not emerge from this state after entering the human body and cause disease. Moreover, a possible impact in food fermentation can be considered if the VBNC cells are responsible for biochemical activities involved in the formation of the final characteristics of the product.

In the field of food microbiology, culture-independent techniques, used as tools to profile microbial ecosystems, have been used more in the food fermentation and food spoilage fields, although, in a few cases, they have also been exploited to study the ecology of food-borne pathogens (Cocolin et al., 2002b; Cocolin and Comi, 2005).

2. Culture-independent methods used in food microbiology

Most of the techniques used for culture independent analysis are PCR-based. After amplification of the nucleic acids extracted directly from the food matrix, the PCR product is subjected to specific analyses that are able to highlight differences in the amplified DNA sequences.

One of the most important aspects that should be considered, in order to properly profile microbial populations in food ecosystems, is the selection of the DNA region that has to be amplified. The target gene has to have two basic characteristics: (i) it should be present in all members of the microbial group that is under consideration, (ii) it should have conserved regions, in which universal primers can be designed, and variable regions, in which differentiation is possible. Genes encoding for ribosomal RNA (rRNA) fall into this category. Various regions of the 16S rRNA coding gene have been used in bacteria, while the 26S rRNA coding gene is commonly the target in yeasts. One important advantage of the use of these two genes is that large sequence databases exist. On the other hand, one drawback of the use of rRNA coding genes is the inherent sequence heterogeneity within the same species, which is the result of multi-copies of the genes with small differences in the sequence (Fogel et al., 1999). These multi-copies often result in multi-signals, which complicate the analysis. The *rpoB* gene, encoding for the β -subunit of the RNA polymerase, has been proposed as an alternative, but its application is still limited (Dahlhoff et al., 2000; Rantsiou et al., 2004).

In post amplification analysis, the goal is to detect DNA sequence heterogeneity. Such a goal is reached using denaturing/temperature gradient gel electrophoresis (D/TGGE) and single strand conformation polymorphism (SSCP) through the study of the electrophoretic mobility of completely or partially denatured PCR products, respectively, or by using restriction endonucleases in terminal restriction fragment length polymorphism (TRFLP).

D/TGGE consists of an electrophoretic separation of PCR products in a polyacrylamide gel containing a gradient of chemical (urea and formamide in DGGE) or physical (temperature in TGGE) denaturants. As the DNA molecule encounters the appropriate denaturant gradient, a sequence-dependent, partial denaturation of the double strand

occurs. This change in the conformation of the DNA structure causes a reduced migration rate of the molecule. When the method is used for microbial profiling, after amplification, the complex mixture of the DNA molecules can be differentiated and characterized. Bands visible in D/TGGE gels represent components of the microbiota. They can be excised and, after re-amplification, can be sequenced in order to obtain the corresponding microbial species. Using these methods, it is possible not only to profile the microbial populations, but also to follow their dynamics over time. It should be noted that these methods are not quantitative (Ercolini, 2004).

In the case of SSCP, the differentiation is based on the mobility of single strands of DNA. Small changes in the sequence can be detected because the single strand may create intrastrand base pairing, which results in loops and folds that give the single strand a unique 3D structure, and this affects its mobility through a gel. In SSCP analysis, the amplified product is denatured to a single-stranded form and subjected to non-denaturing polyacrylamide gel electrophoresis. In the last few years, SSCP methods have been based on amplification with fluorescein-labelled primers and detection of the signals by fluorescence. When SSCP is used to profile a complex microbial ecosystem, a robust database should be created in order to be able to identify each single component by comparing the retention time of each signal with a reference time in the database. If matching does not occur, identification cannot be obtained (Hayashi, 1992).

Finally, in T-RFLP, one of the PCR primers is labelled with fluorescent dye and used to amplify a selected region of a gene of interest by means of PCR. The resulting PCR fragment is digested with one (or more) restriction endonuclease(s) and the resulting fragments are separated by means of an automated DNA analyzer. Microbial diversity in a community can be estimated by analysing the number and peak heights of patterns. T-RFLP is an effective tool for characterizing the dynamic changes that occur in complex microbial ecosystems over time. However, the technique is best suited for microbial communities with low to intermediate richness (Sibley et al., 2012).

These PCR-based assays all suffer from amplification bias. Appropriate primer selection is essential, but it must be noted that a perfect universal primer set does not exist for community profiling. Usually they are reproducible, however low number populations signals may not always be detected if multiple runs of the same sample are carried out. Regarding the sensitivity it has been recognized that these methods are able to detect as low as 1% of the total community, however this limit depends on the composition of the microbial ecosystem and on the detection strategy (gel based or by using fluorescent dyes in capillary electrophoresis). For DGGE analysis the limit of detection has been described to be about 10^3 colony forming units (cfu)/ml or g (Cocolin et al., 2001a, 2001b).

Among the culture-independent methods, one of the few that do not rely on PCR amplification is fluorescence in situ hybridization (FISH). This technique is based on the hybridization of fluorescein-labelled probes to specific sequences of the rRNA. Target cells are immobilized on a microscope glass and then subjected to a permeabilization step in order to allow the probe to penetrate into the cell. After hybridization, the results are visualized under a UV microscope (Bottari et al., 2006). FISH has not been used intensively in food microbiology, although it has the great potential of being able to localize microbial populations in a solid food matrix (Ercolini et al., 2003).

Considering the wide application of DGGE and its extensive literature in the field of food microbiology, this review will be focused on this method.

3. DGGE applications in food microbiology: a temporal evolution

As reported above, DGGE is the culture-independent technique that has been used most often in food microbiology. The first paper to exploit the potential of DGGE, by Muyzer et al., was published in

1993. They investigated the microbial ecology of mats taken from different depths and bacterial biofilms isolated from aerobic and anaerobic wastewater treatment reactors. Only in the late 90s, was DGGE introduced into food microbiology and since then an extensive number of studies, exploiting DGGE as a method to profile microbial ecology in food, have been reported (Table 1). It is interesting to note that DGGE has been applied to all areas of food microbiology, such as food fermentation, food spoilage and food safety, the former being the richest in terms of scientific literature. DGGE is most suitable for the study of the microbial ecology of spontaneous fermentations. Wine, meat and meat products, milk and dairy products are the most frequently studied kinds of food, both in terms of fermentation and spoilage processes, while sourdough and vegetables have been investigated to a lesser extent using culture-independent methods. DGGE has been the subject of several review papers, some of which have focused on its general aspects and applications (Ercolini, 2004), while others have dealt with specific reviews in the dairy sector (Jany and Barbier, 2008; Quigley et al., 2011), meat fermentations (Rantsiou and Cocolin, 2006; Cocolin et al., 2011b), grape surface in relation to wine production (Barata et al., 2012), wine and beer fermentation (Cocolin et al., 2011a; Bokulich et al., 2012) and spoilage of meat (Doulgeraki et al., 2012).

A search conducted in Scopus (www.scopus.com) in December 2012, using DGGE and food as keywords, resulted in more than 400 hits, with the first one dating back to 1999 (Ampe et al., 1999). The trend presented in Fig. 1 has been obtained considering the number of papers published each year, from 1998 up to now. As it can be observed, three time spans can be distinguished. Period 1, from the late 90s to 2004, is characterized by a slight, but steady increase in the number of papers published. This is followed by the second period, from 2005 to 2008, in which the studies exploiting DGGE almost triplicate in just 4 years. The number of DGGE papers reaches a peak in the third period (2009–present), in 2010, after which a decrease can be observed.

Analysing the temporal evolution of the use of DGGE in food microbiology, it is worth noticing that a correlation exists between the subjects considered in the papers and their distribution in the three time spans described above. In the first years of application, DGGE was mostly used to study well established food fermentations in industrialized countries. Fermented sausage (Cocolin et al., 2001a), cheese (Ercolini et al., 2001) and wine (Cocolin et al., 2001b) were the first products to be investigated, although studies focusing on the ecology of Mexican pozol (Ampe et al., 1999; Ben Omar and Ampe, 2000) were also published. The portfolio of food investigated by means of DGGE expanded in the following years, when studies on sourdough (Meroth et al., 2003a), whisky (van Beek and Priest, 2002) and raw milk ecology (Lafarge et al., 2004) became available.

A remarkable increase in the number of DGGE papers can be observed for the following time period. This could be correlated to an intensification of the studies on the fermented products described above (especially fermented sausage and cheese), but also to the exploitation of DGGE to follow the dynamic changes that occur during food spoilage. The first examples of DGGE application to investigate spoilage organisms date back to 2004, when two papers, one focusing on the late blowing of cheese (Cocolin et al., 2004a) and the other on fresh sausage storage at refrigeration temperatures (Cocolin et al., 2004b) were published. An important contribution to the increasing trend of DGGE papers in this period was given by the spoilage studies from 2006, when, for the first time, DGGE was applied to investigate the microbiota of fresh meat during refrigerated storage under different packaging conditions (Ercolini et al., 2006).

The growing trend observed in Fig. 1 until 2010 could be due to the increase in studies in which DGGE was used as a tool to study the ecology of different kinds of fermented food from the ones described above. Kimchi (Lee et al., 2005) and cocoa (Nielsen et al., 2005) were among the first products to be investigated, and these have been followed in recent years by soybean-based foods (Kim et al., 2009, 2010a, 2010b; Park

et al., 2009; Lee et al., 2010). The decrease in the number of studies should be considered carefully, and it is necessary to consider that information related to the 2012 papers will only become available at the beginning of 2013.

4. What have we learnt from the application of DGGE in food microbiology?

The results obtained by applying DGGE as a culture-independent method to food fermentation have generally confirmed previous knowledge obtained through traditional microbiological methods. Members of lactic acid bacteria (LAB) were found to be the main microorganisms active in food fermentation, but also involved in spoilage processes together with *Pseudomonas* spp. and *Enterobacteriaceae* (Table 1). From this point of view, the novelties introduced by the application of DGGE are related not so much to the discovery of new microbial species, but to a new way of globally investigating the ecology of food during microbial transformations.

4.1. DNA versus RNA DGGE analysis

One of the potentials of DGGE is that it offers the possibility of performing ecological studies that target both nucleic acids, that is, DNA and RNA. It should be underlined that these two molecules have completely different biological meanings, since DNA contains hereditary messages, and RNA has a direct involvement in its translation into proteins. Moreover, DNA shows remarkable stability in the environment, as can be seen from the recovery of DNA, and the successful amplification by PCR, from archaeological and paleontological samples, which can be thousands of years old (Landweber, 1999), while RNA, and more specifically messenger RNA (mRNA), persists for short periods of time in actively growing bacteria cells, with an average half-life measured in minutes (Arraiano et al., 1998). Studying the DNA of a microbial ecosystem in ecological studies allows the microbial ecology and diversity to be defined, while RNA analysis is able to better highlight the microbial populations that are metabolically active, and thereby contribute to the microbial process. Since the number of intact ribosomes approximately reflects the rate of protein synthesis, ribosomal RNA (rRNA) can be used as a marker for general metabolic activity (Gosalbes et al., 2011), although it must be accepted that these molecules are characterized by a much higher level of protection, than mRNA (sometimes even weeks). Another strategy to detect viable population is the use of ethidium monoazide (EMA) and propidium monoazide (PMA), DNA-intercalating agents able to selectively penetrate the membranes of dead cells and form stable DNA monoadducts upon photolysis, resulting in DNA which cannot be amplified by PCR. While this approach has been frequently used to differential live and dead cells of pathogenic microorganisms (Rudi et al., 2005), its performances in complex microbial ecosystems has not been tested.

RNA has not been used extensively in DGGE studies, but, when targeted, it has allowed some new evidence to be highlighted, especially in food fermentation. In dairy fermentation, the differentiation between starter LAB (SLAB), mainly *Lactococcus* spp., *Streptococcus thermophilus* and several *Lactobacillus* spp., and non-starter LAB (NSLAB), belonging to *Lactobacillus* spp., *Leuconostoc* spp. and *Pediococcus* spp. (Fox et al., 2004), is generally scientifically accepted. The former are responsible for the acidification of milk and curd in the early stages of cheese manufacturing, while NSLAB are considered as secondary microbiota, associated with the organoleptic development of cheese during ripening. Studies conducted on different types of cheese (Dolci et al., 2008, 2010; Rantsiou et al., 2008) have confirmed this switch in LAB populations through traditional methods. However, they have also highlighted the presence of stable signals of *L. lactis* and *S. thermophilus* at an RNA level in the late stages of ripening. This new information leads to the conclusion that SLAB are not only

Table 1
Studies exploiting the DGGE technique to study the microbial ecology of various food products.

Food products	Country	Microbial species identified	Target gene(s)	Reference
<i>Milk and dairy products</i>				
Flemish artisan raw milk Gouda-type cheese	Belgium	<i>Enterococcus faecalis</i> , <i>Lactobacillus parabucheri</i> , <i>Lactobacillus gallinarum</i>	16S rRNA gene	Van Hoorde et al. (2008)
Gouda-type cheeses		<i>Lactococcus lactis</i> , <i>Lactobacillus plantarum</i> , <i>Lactobacillus casei</i> , <i>Leuconostoc pseudomesenteroides</i>	16S rRNA gene	Van Hoorde et al. (2010)
Capo Verde cheese	Capo Verde Islands	<i>Lactococcus lactis</i> subsp. <i>lactis</i> , <i>Lactobacillus helveticus</i> , <i>Kocuria rhizophila</i> , <i>Escherichia coli</i> , <i>Delphinella strobiligena</i> , <i>Saccharomyces cerevisiae</i> , <i>Filobasidium elegans</i>	16S and 26S rRNA genes	Alessandria et al. (2010)
Yoghurt	China	<i>Streptococcus thermophilus</i> , <i>Lactobacillus delbrueckii</i> , <i>Lactobacillus crispatus</i> , <i>Lactobacillus rhamnosus</i>	16S rRNA gene	Ma et al. (2009)
Calenzana cheese	Corsica	<i>Lactococcus lactis</i> ssp. <i>lactis</i> , coryneform bacteria	16S rRNA gene	Casalta et al. (2009)
Domiaty cheese	Egypt	<i>Leuconostoc mesenteroides</i> , <i>Lactococcus garvieae</i> , <i>Aerococcus viridans</i> , <i>Lactobacillus versmoldensis</i> , <i>Pediococcus inopinatus</i> , <i>Lactococcus lactis</i> , <i>Kocuria rhizophila</i> , <i>Kocuria kristinae</i> , <i>Arthrobacter</i> sp., <i>Brachybacterium tyrofermentans</i>	16S rRNA gene	El-Baradei et al. (2007)
Zabady fermented milk		<i>Streptococcus thermophilus</i> , <i>Lactococcus garvieae</i> , <i>Lactobacillus raffinolactis</i> , <i>Leuconostoc citreum</i>	16S rRNA gene	El-Baradei et al. (2008)
Different cheeses	France	<i>Lactococcus lactis</i> , <i>Streptococcus thermophilus</i> , <i>Lactobacillus buchneri</i> , <i>Corynebacterium variabile</i> , <i>Lactobacillus plantarum</i>	16S rRNA gene	Ogier et al. (2004)
Raw milk during refrigeration		<i>Lactococcus lactis</i> , <i>Staphylococcus warneri</i> , <i>Staphylococcus epidermidis</i> , <i>Klebsiella pneumoniae</i> , <i>Kocuria rosea</i> , <i>Listeria</i> spp., <i>Arthrobacter</i> sp.	16S rRNA gene	Lafarge et al. (2004)
Fermented milk	Ghana	<i>Lactobacillus acidophilus</i> , <i>Streptococcus salivarius</i> , <i>Lactobacillus delbrueckii</i> subsp. <i>delbrueckii</i> , <i>Streptococcus thermophilus</i>	16S rRNA gene	Obodai and Dodd (2006)
Feta cheese	Greece	<i>Streptococcus thermophilus</i> , <i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i> , <i>Kluyveromyces lactis</i> , <i>Pichia membranifaciens</i> , <i>Pichia fermentans</i>	16S and 26S rRNA genes	Rantsiou et al. (2008)
Caciocavallo Silano	Italy	<i>Lactobacillus delbrueckii</i> , <i>Lactobacillus helveticus</i> , <i>Streptococcus thermophilus</i> , <i>Lactococcus lactis</i>	16S rRNA gene	Ercolini et al. (2008)
Caciotta cheese		<i>Leuconostoc mesenteroides</i> , <i>Lactobacillus fermentum</i> , <i>Lactobacillus sakei/curvatus</i>	16S rRNA gene	Aquilanti et al. (2011)
Castelmagno cheese		<i>Lactobacillus plantarum</i> , <i>Streptococcus agalactiae</i> , <i>Lactococcus lactis</i> subsp. <i>lactis</i> , <i>Lactococcus lactis</i> subsp. <i>cremoris</i> , <i>Lactobacillus kefirifaciens</i>	16S rRNA gene	Dolci et al. (2008)
Fontina cheese rinds		<i>Lactococcus lactis</i> , <i>Lactobacillus helveticus</i> , <i>Streptococcus agalactiae</i> , <i>Lactococcus lactis</i> subsp. <i>lactis</i> , <i>Streptococcus thermophilus</i> , <i>Arthrobacter nicotianae</i> , <i>Brevibacterium casei</i> , <i>Corynebacterium glutamicum</i> , <i>Debaryomyces hansenii</i> , <i>Candida sake</i>	16S and 26S rRNA genes	Dolci et al. (2009)
Fontina cheese		<i>Streptococcus thermophilus</i> , <i>Enterococcus faecium</i> , <i>Lactococcus lactis</i> , <i>Macrocococcus caseolyticus</i> , <i>Chryseobacterium</i> spp.	16S rRNA gene	Giannino et al. (2009)
Gorgonzola rinds		<i>Arthrobacter</i> sp., <i>Staphylococcus</i> sp.	16S rRNA gene	Cocolin et al. (2009a)
Parmigiano Reggiano cheese		<i>Lactobacillus fermentum</i> , <i>Lactobacillus casei</i> , <i>Lactobacillus paracasei</i> , <i>Lactobacillus helveticus</i> , <i>Lactobacillus delbrueckii</i> subsp. <i>Lactis</i>	16S rRNA gene	Gala et al. (2008)
Pecorino Siciliano cheese		<i>Streptococcus bovis</i> , <i>Lactococcus lactis</i> , <i>Streptococcus thermophilus</i> , <i>Enterococcus durans</i> , <i>Lactobacillus rhamnosus</i> , <i>Lactobacillus casei</i>	16S rRNA gene 16S rRNA gene	Randazzo et al. (2006) Randazzo et al. (2008)
Ragusano Cheese		<i>Streptococcus thermophilus</i> , <i>Lactococcus lactis</i> , <i>Lactobacillus delbrueckii</i> , <i>Enterococcus faecium</i>	16S rRNA gene	Licitra et al. (2007)

Table 1 (continued)

Food products	Country	Microbial species identified	Target gene(s)	Reference
		<i>Leuconostoc</i> sp., <i>Lactococcus lactis</i> , <i>Macrocooccus caseolyticus</i> , <i>Streptococcus</i> <i>thermophilus</i> , <i>Lactobacillus delbrueckii</i> , <i>Lactobacillus fermentum</i>	16S rRNA gene	Randazzo et al. (2002)
Raw milk		<i>Candida</i> spp., <i>Kluyveromyces</i> spp.	26S rRNA gene	Cocolin et al. (2002a)
Robiola di Roccaverano		<i>Lactococcus lactis</i> subsp. <i>lactis</i> , <i>Geotrichum</i> spp., <i>Kluyveromyces lactis</i>	16S and 26S rRNA genes	Bonetta et al. (2008)
Surface microbiota of Taleggio, Gorgonzola, Casera, Scimudin and Formaggio di Fossa		<i>Psychrobacter celer</i> , <i>Psychrobacter</i> <i>aquimaris</i> , <i>Micrococcus luteus</i> , <i>Staphylococcus equorum</i>	16S rRNA gene	Fontana et al. (2010)
Italian cheeses				
Taleggio cheese		<i>Arthrobacter</i> spp., <i>Brevibacterium</i> spp., <i>Pseudoalteromonas agarivorans</i> , <i>Brevibacterium linens</i>	16S rRNA gene	Feligini et al. (2012)
Tosela cheese		<i>Lactobacillus paracasei</i> , <i>Streptococcus</i> <i>macedonicus</i> , <i>Lactobacillus rhamnosus</i>	16S rRNA gene	Settanni et al. (2011)
Water- <i>Buffalo</i> Mozzarella Cheese		<i>Streptococcus thermophilus</i> , <i>Lactococcus lactis</i> , <i>Lactobacillus</i> <i>delbrueckii</i> , <i>Lactobacillus crispatus</i>	16S rRNA gene	Ercolini et al. (2004)
Tarag cheese	Mongolia	<i>Lactobacillus helveticus</i> , <i>Lactococcus lactis</i> subsp. <i>lactis</i> , <i>Lactobacillus casei</i>	16S rRNA gene	Liu et al. (2012)
Oscypek cheese	Poland	<i>Lactococcus lactis</i> , <i>Lactobacillus</i> <i>plantarum</i> , <i>Leuconostoc citreum</i> , <i>Streptococcus vestibularis</i> , <i>Debaryomyces</i> <i>hansenii</i> , <i>Candida pararugosa</i>	16S and 26S rRNA genes	Alegría et al. (2012)
Bukuljac cheese	Serbia	<i>Leuconostoc mesenteroides</i>	16S rRNA gene	Nikolic et al. (2008)
Cabrales Spanish blueveined cheese	Spain	<i>Lactococcus garviae</i> , <i>Lactococcus</i> <i>lactis</i> subsp. <i>lactis</i> , <i>Lactobacillus</i> <i>raffinolactis</i> , <i>Penicillium roqueforti</i> , <i>Geotrichum candidum</i>	16S and 26S rRNA genes	Flórez and Mayo (2006)
Casín cheese		<i>Streptococcus thermophilus</i> , <i>Lactococcus</i> <i>lactis</i> , <i>Streptococcus parauberis</i> , <i>Lactobacillus plantarum</i> , <i>Geotrichum</i> <i>candidum</i> , <i>Kluyveromyces lactis</i>	16S and 26S rRNA genes	Alegría et al. (2009)
Kefir grains	Taiwan	<i>Lactobacillus kefiranofaciens</i> , <i>Lactobacillus kefiri</i> , <i>Lactococcus lactis</i>	16S rRNA gene	Chen et al. (2008)
Taiwanese ropy fermented milk		<i>Lactococcus lactis</i> subsp. <i>lactis</i> , <i>Leuconostoc mesenteroides</i> subsp. <i>mesenteroides</i> , <i>Lactobacillus</i> <i>fermentum</i>	16S rRNA gene	Wang et al. (2011b)
Tibetan Kefir	Tibet	<i>Pseudomonas</i> sp., <i>Leuconostoc</i> <i>mesenteroides</i> , <i>Lactobacillus helveticus</i> , <i>Lactobacillus kefiranofaciens</i> , <i>Lactococcus</i> <i>lactis</i> , <i>Lactobacillus kefiri</i> , <i>Lactobacillus</i> <i>casei</i> , <i>Kazachstania unispora</i> , <i>Kluyveromyces marxianus</i> , <i>Saccharomyces cerevisiae</i>	16S and 26S rRNA genes	Zhou et al. (2009)
Kefir grains and kefir beverages	Turkey	<i>Lactobacillus kefiranofaciens</i> , <i>Lactococcus lactis</i>	16S rRNA gene	Kesmen and Kacmaz (2011)
<i>Fermented meats</i>				
Fermented sausages	Argentina	<i>Lactobacillus sakei</i> , <i>Lactobacillus</i> <i>plantarum</i> , <i>Staphylococcus</i> <i>saprophyticus</i>	16S rRNA gene	Fontana et al. (2005a)
		<i>Lactobacillus plantarum</i> , <i>Lactobacillus</i> <i>sakei</i> , <i>Staphylococcus saprophyticus</i> , <i>Corynebacterium variabilis</i> , <i>Brochothrix</i> <i>thermophacta</i>	16S rRNA gene	Fontana et al. (2005b)
	Italy	<i>Lactobacillus plantarum</i> , <i>Staphylococcus</i> <i>carneus</i> , <i>Lactobacillus curvatus</i>	16S rRNA gene	Cocolin et al. (2006b)
		<i>Lactobacillus plantarum</i> , <i>Lactobacillus</i> <i>curvatus</i> , <i>Lactobacillus sakei</i>	16S rRNA gene	Cocolin et al. (2001a)
		<i>Penicillium farinosum</i> , <i>Debaryomyces</i> <i>hansenii</i> , <i>Penicillium viridicatum</i> , <i>Mucor racemosus</i>	26S rRNA gene	Cocolin et al. (2006a)
		<i>Enterococcus faecalis</i> , <i>Pediococcus</i> <i>acidilactici</i> , <i>Lactobacillus rhamnosus</i> , <i>Enterococcus faecium</i> , <i>Enterococcus</i> <i>durans</i> , <i>Lactobacillus sakei</i>	16S rRNA gene	Gazzola et al. (2012)
		<i>Lactobacillus curvatus</i> , <i>Lactobacillus</i> <i>sakei</i> , <i>Brochothrix thermosphacta</i> , <i>Macrocooccus caseolyticus</i> , <i>Staphylococcus xylosum</i>	16S rRNA gene	Rantsiou et al. (2005)
Fermented sausages	Italy	<i>Lactobacillus plantarum</i> , <i>Lactobacillus</i> <i>curvatus</i> , <i>Lactobacillus sakei</i> ,	16S and 26S rRNA genes	Aquilanti et al. (2007)

(continued on next page)

Table 1 (continued)

Food products	Country	Microbial species identified	Target gene(s)	Reference
		<i>Lactococcus lactis</i> ssp. <i>lactis</i> , <i>Staphylococcus xylosum</i> , <i>Debaryomyces hansenii</i>	16S rRNA gene	Cocolin et al. (2009b)
Soppressata of Vallo di Diano sausage		<i>Lactobacillus curvatus</i> , <i>Lactobacillus sakei</i> , <i>Lactobacillus paraplantarum</i> , <i>Lactobacillus algidus</i> , <i>Leuconostoc mesenteroides/pseudomesenteroides</i> , <i>Staphylococcus saprophyticus</i>	16S and 26S rRNA genes	Villani et al. (2007)
Alheira: traditional Portuguese fermented sausage	Portugal	<i>Staphylococcus xylosum</i> , <i>Staphylococcus succinus</i> , <i>Staphylococcus equorum</i> , <i>Lactobacillus sakei</i> , <i>Lactobacillus curvatus</i> , <i>Debaryomyces hansenii</i>	16S rRNA gene	Albano et al. (2008)
Fermented ham	Taiwan	<i>Brochothrix thermosphacta</i> , <i>Lactobacillus sakei</i> , <i>Leuconostoc lactis</i> , <i>Streptococcus lutetiensis</i> , <i>Lactobacillus curvatus</i> , <i>Lactobacillus brevis</i>	16S rRNA gene	Tu et al. (2010)
		<i>Lactobacillus sakei</i> , <i>Staphylococcus saprophyticus</i> , <i>Lactobacillus mesenteroides</i> , <i>Carnobacterium divergens</i> , <i>Brochothrix thermosphacta</i>		
Cereal-based foods				
Sourdoughs	Belgium	<i>Lactobacillus spicheri</i> , <i>Lactobacillus plantarum</i> , <i>Lactobacillus sanfranciscensis</i>	16S rRNA gene	Scheirlinck et al. (2009)
		<i>Lactobacillus frumenti</i> , <i>Lactobacillus panis</i> , <i>Lactobacillus pontis</i> , <i>Lactobacillus curvatus</i>	16S rRNA gene	De Vuyst and Vancanneyt (2007)
		<i>Lactobacillus sanfranciscensis</i> , <i>Lactobacillus paralimentarius</i> , <i>Lactobacillus plantarum</i> , <i>Lactobacillus pontis</i> , <i>Acetobacter</i> sp.	16S rRNA gene	Scheirlinck et al. (2008)
Rice sourdough	Germany	<i>Lactobacillus plantarum</i> , <i>Lactobacillus fermentum</i> , <i>Lactobacillus kimchii</i> , <i>Lactobacillus gallinarum</i> , <i>Lactobacillus pontis</i>	16S rRNA gene	Meroth et al. (2004)
Sourdough		<i>Lactobacillus sanfranciscensis</i> , <i>Lactobacillus crispatus</i> , <i>Lactobacillus pontis</i> , <i>Lactobacillus johnsonii</i> , <i>Lactobacillus mindensis</i>	16S rRNA gene	Meroth et al. (2003a)
		<i>Saccharomyces cerevisiae</i> , <i>Candida humilis</i> , <i>Dekkera bruxellensis</i>	26S rRNA gene	Meroth et al. (2003b)
Panettone	Italy	<i>Lactobacillus sanfranciscensis</i> , <i>Lactobacillus brevis</i> , <i>Candida humilis</i>	16S and 26S rRNA genes	Garofalo et al. (2008)
Sourdough		<i>Saccharomyces cerevisiae</i> , <i>Kazachstania unispora</i> , <i>Saccharomyces bayanus</i> / <i>Kazachstania</i> sp., <i>Candida humilis</i>	26S rRNA gene	Minervini et al. (2012)
		<i>Lactobacillus sanfranciscensis</i> , <i>Lactobacillus fermentum</i>	16S rRNA gene	Randazzo et al. (2005)
		<i>Lactobacillus plantarum</i> , <i>Lactobacillus sanfranciscensis</i> , <i>Lactobacillus rossiae</i>	16S rRNA gene	Settanni et al. (2006)
Ting: fermented sorghum	South Africa	<i>Weissella cibaria</i> , <i>Lactobacillus curvatus</i> , <i>Lactococcus lactis</i>	16S rRNA gene	Madoroba et al. (2011)
Maize fermentation	Mexico, Congo and Benin	<i>Lactobacillus plantarum</i> , <i>Lactobacillus delbrueckii</i> , <i>Lactobacillus fermentum</i>	16S rRNA gene PCR-DGGE	Ampe and Miambi (2000)
Fermented cassava	Congo	<i>Lactobacillus plantarum/pentosus</i> , <i>Lactobacillus plantarum/sanfrancisco</i> , <i>Lactobacillus fermentum</i> , <i>Lactobacillus delbrueckii</i> , <i>Clostridium acetobutylicum</i> / <i>butyricum</i>	16S rRNA gene	Miambi et al. (2003)
Mexican fermented and maize dough pozol	Mexico	<i>Streptococcus bovis</i> , <i>Enterococcus saccharolyticus</i> , <i>Lactobacillus fermentum</i> , <i>Lactobacillus plantarum</i> , <i>Exiguobacterium aurantiacum</i>	16S rRNA gene	Ben Omar and Ampe (2000)
Pozol		<i>Lactobacillus plantarum</i> , <i>Lactobacillus fermentum</i> , <i>Streptococcus bovis</i> , <i>Weissella paramesenteroides</i>	16S rRNA gene	Ampe et al. (1999)
Fresh and fermented vegetables				
Fuzhuan brick-tea	China	<i>Debaryomyces</i> spp., <i>Eurotium</i> spp., <i>Aspergillus niger</i>	26S rRNA gene	Xu et al. (2011)
Fresh cut salad	Italy	<i>Pseudomonas siringae</i> , <i>Pantoea agglomerans</i> , <i>Pseudomonas rhodesiae</i> , <i>Erwinia persicinus</i>	16S rRNA gene	Randazzo et al. (2009)

Table 1 (continued)

Food products	Country	Microbial species identified	Target gene(s)	Reference
Sunki	Japan	<i>Lactobacillus fermentum</i> , <i>Lactobacillus plantarum</i> , <i>Lactobacillus delbrueckii</i>	16S rRNA gene	Endo et al. (2008)
Kimchi	Korea	<i>Halococcus</i> spp., <i>Natronococcus</i> spp., <i>Natrialba</i> spp., <i>Lodderomyces</i> spp., <i>Trichosporon</i> spp.	16S rRNA gene	Chang et al. (2008)
		<i>Weissella confusa</i> , <i>Leuconostoc citreum</i> , <i>Lactobacillus sakei</i> , <i>Lactobacillus curvatus</i>	16S rRNA gene	Lee et al. (2005)
Dongchimi	South Korea	<i>Lactobacillus algidus</i> , <i>Lactobacillus plantarum</i>	16S rRNA gene	Park et al. (2008)
Olives	Spain	<i>Gordonia</i> sp., <i>Pseudomonas</i> sp., <i>Sphingomonas</i> sp., <i>Halosarcina pallida</i> , <i>Lactobacillus</i> sp., <i>Saccharomyces cerevisiae</i> , <i>Lactobacillus pentosus</i> / <i>plantarum</i> , <i>Lactobacillus vaccinostrercus</i> / <i>suebicus</i> , <i>Candida cf. apicola</i>	16S and 26S rRNA genes	Abriouel et al. (2011)
Wine, vinegar and other beverages				
Caxiri, alcoholic beverage	Brazil	<i>Paenibacillus</i> sp., <i>Bacillus subtilis</i> , <i>Lactobacillus fermentum</i> , <i>Saccharomyces cerevisiae</i> , <i>Pichia membranifaciens</i> , <i>Rhodotorula mucilaginosa</i>	16S and 26S rRNA genes	Santos et al. (2012)
Palm wine	Cameroon	<i>Saccharomyces cerevisiae</i> , <i>Saccharomycodes ludwigii</i> , <i>Zygosaccharomyces bailii</i> , <i>Hanseniaspora uvarum</i> , <i>Candida parapsilopsis</i> , <i>Candida fermentati</i> , <i>Pichia fermentans</i>	26S rRNA gene	Stringini et al. (2009)
Hong Qu glutinous rice wine	China	<i>Janthinobacterium lividum</i> , <i>Bacillus subtilis</i> , <i>Bacillus amyloliquefaciens</i> , <i>Saccharomycopsis fibuligera</i> , <i>Rhizopus oryzae</i> , <i>Aspergillus flavus</i>	16S rRNA and 18S genes	Lv et al. (2012)
Sauternes wines	France	<i>Saccharomyces cerevisiae</i> , <i>Candida stellata</i> , <i>Hanseniaspora uvarum</i> , <i>Botrytis cinerea</i>	26S rRNA gene	Divol and Lonvaud-Funel, (2005)
Botrytis-affected wine fermentations	Greece	<i>Metschnikowia pulcherrima</i> , <i>Zygosaccharomyces bailii</i> , <i>Issatchenkia</i> spp., <i>Botrytis cinerea</i>	26S rRNA gene	Nisioutou et al. (2007)
Botrytis-affected grapes and wine fermentations		<i>Klebsiella oxytoca</i> , <i>Citrobacter freundii</i> , <i>Enterobacter</i> spp., <i>Erwinia</i> sp., <i>Pantoea dispersa</i> , <i>Tatumella ptyseos</i> on grapes.		
		<i>Lactobacillus plantarum</i> and <i>Enterobacter ludwigii</i> during fermentations	16S rRNA gene	Nisioutou et al., 2011
Balsamic vinegar	Italy	<i>Acetobacter pasteurianus/acetii</i> , <i>Acetobacter pasteurianus</i> , <i>Gluconacetobacter europaeus</i>	16S rRNA gene 16S rRNA gene	De Vero et al. (2006) Gullo et al. (2009)
Erbaluce wine		<i>Candida zemplinina</i> , <i>Metschnikowia fructicola</i> , <i>Hanseniaspora uvarum</i> , <i>Saccharomyces cerevisiae</i>	26S rRNA gene	Rantsiou et al. (2013)
Picolit wine		<i>Saccharomyces cerevisiae</i> , <i>Aureobasidium pullulans</i> , <i>Candida zemplinina</i>	26S rRNA gene	Urso et al. (2008)
Vinegard	Japan	<i>Aspergillus oryzae</i> , <i>Saccharomyces</i> sp., <i>Lactobacillus acetotolerance</i> , <i>Acetobacter pasteurianus</i>	16S and 26S rRNA genes	Haruta et al. (2006)
Takju: Korean rice wines	Korea	<i>Lactobacillus paracasei</i> , <i>Lactobacillus plantarum</i> , <i>Leuconostoc pseudomesenteroides</i> , <i>Lactobacillus harbinensis</i> , <i>Lactobacillus parabuchneri</i>	16S rRNA gene	Kim et al. (2010a, 2010b)
Mezcal, alcoholic beverage	Mexico	<i>Pediococcus parvulus</i> , <i>Lactobacillus brevis</i> , <i>Lactobacillus composti</i> , <i>Lactobacillus parabuchneri</i> , <i>Lactobacillus plantarum</i>	16S rRNA gene	Narváez-Zapata et al. (2010)
Fruit juice	South Africa	<i>Alicyclobacillus acidoterrestris</i> , <i>Alicyclobacillus pomorum</i>	16S rRNA gene	Duvenage et al. (2007)
Makgeolli	South Korea	<i>Lactobacillus crustorum</i> , <i>Lactobacillus brevis</i> , <i>Lactobacillus plantarum</i>	16S rRNA gene	Kim et al. (2012)
Tempranillo wines	Spain	<i>Oenococcus oeni</i> , <i>Gluconobacter oxydans</i> , <i>Asaia siamensis</i>	16S rRNA gene	Ruiz et al. (2010)
Kava beverages	Unites States	<i>Weissella soli</i> , <i>Lactobacillus</i> spp., <i>Lactococcus lactis</i>	16S rRNA gene	Dong et al. (2011)
Botrytis-affected wine fermentations		<i>Hanseniaspora uvarum</i> , <i>Hanseniaspora osmophila</i> , <i>Candida zemplinina</i> , <i>Lachancea thermotolerans</i> , <i>Saccharomyces cerevisiae</i>	26S rRNA gene	Mills et al. (2002)

(continued on next page)

Table 1 (continued)

Food products	Country	Microbial species identified	Target gene(s)	Reference
Sweet wine fermentation		<i>Metschnikowia</i> sp., <i>Botrytis cinerea</i> , <i>Pichia anomala</i> , <i>Saccharomyces cerevisiae</i>	26S rRNA gene	Cocolin et al. (2001b)
<i>Fish products</i>				
Chum salmon sauce	Japan	<i>Zygosaccharomyces rouxii</i> , <i>Candida versatilis</i> , <i>Aspergillus oryzae</i>	26S rRNA gene	Yoshikawa et al. (2010)
Aji-narezushi and iwashi-nukazuke		<i>Lactobacillus acidipiscis</i> , <i>Lactobacillus versmoldensis</i> , <i>Lactobacillus plantarum</i> , <i>Tetragenococcus muriaticus</i> , <i>Tetragenococcus halophilus</i>	16S rRNA gene	An et al. (2010)
Sea food	Korea	<i>Sulfolobus</i> sp., <i>Thermocodium</i> sp., <i>Lactobacillus</i> sp., <i>Weissella</i> sp., <i>Salinivibrio</i> sp.	16S rRNA gene	Roh et al. (2010)
Philippine fermented food products	Philippine	<i>Lactobacillus fermentum</i> , <i>Lactobacillus plantarum</i> , <i>Lactobacillus panis</i> , <i>Lactobacillus pontis</i> , <i>Acetobacter pomorum</i> , <i>Acetobacter ghanensis</i>	16S rRNA gene	Dalmacio et al. (2011)
<i>Cocoa and african foods</i>				
Tayohounta: natural fermentation of baobab seed kernels	Benin	<i>Bacillus licheniformis</i> , <i>Bacillus pumilus</i> , <i>Bacillus subtilis</i> , <i>Bacillus thermoamylovorans</i> , <i>Lactobacillus fermentum</i>	16S rRNA gene	Chadare et al. (2011)
Cocoa beans	Brazil, Ecuador, Ivory Coast, Malaysia	<i>Hanseniaspora</i> sp., <i>Pichia kudriavzevii</i> , <i>Saccharomyces cerevisiae</i> , <i>Hyphopichia burtonii</i> , <i>Meyerozyma caribbica</i>	26S rRNA gene	Papalexandratou and De Vuyst (2011)
	Ghana	<i>Hanseniaspora guilliermondii</i> , <i>Candida krusei</i> , <i>Candida zemplinina</i> , <i>Pichia membranifaciens</i>	26S rRNA gene	Nielsen et al. (2005)
		<i>Hanseniaspora guilliermondii</i> , <i>Candida diversa</i> , <i>Candida zemplinina</i> , <i>Pichia membranifaciens</i> , <i>Issatchenkia orientalis</i> , <i>Lactobacillus fermentum</i> , <i>Bacillus licheniformis</i> , <i>Acetobacter pasteurianus</i> , <i>Leuconostoc pseudoficulneum</i>	16S and 26S rRNA genes	Nielsen et al. (2007)
		<i>Lactobacillus fermentum</i> , <i>Lactobacillus pseudomesenteroides</i> , <i>Lactobacillus plantarum</i> , <i>Weissella hanaensis</i>	16S rRNA gene	Camu et al. (2007)
Cocoa fermentation		<i>Lactobacillus fermentum</i> , <i>Leuconostoc pseudomesenteroides</i> , <i>Leuconostoc pseudoficulneum</i> , <i>Acetobacter pasteurianus</i> , <i>Saccharomycopsis crateagensis</i> , <i>Hanseniaspora guilliermondii</i> , <i>Candida zemplinina</i>	16S and 26S rRNA genes	Nielsen et al. (2008)
Processing of cocoa beans into cocoa powder	Netherlands	<i>Bacillus licheniformis</i> , <i>Bacillus subtilis</i>	16S rRNA gene	Lima et al. (2012)
Cassava for gari production	Nigeria	<i>Issatchenkia scutulata</i> , <i>Candida rugopelliculosa</i> , <i>Candida maritima</i> , <i>Zygosaccharomyces rouxii</i> , <i>Galactomyces geotricum</i>	18S rRNA gene	Oguntoyinbo (2011)
Cereal foods: kunu-zaki and ogi		<i>Weissella confusa</i> , <i>Lactobacillus fermentum</i> , <i>Lactobacillus amylolyticus</i> , <i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i> , <i>Bacillus</i> sp., <i>Lactococcus lactis</i> subsp. <i>lactis</i>	16S rRNA gene	Oguntoyinbo et al. (2011)
Ugandan Ghee	Uganda	<i>Lactobacillus helveticus</i> , <i>Lactobacillus plantarum</i> , <i>Bifidobacterium</i> sp., <i>Enterococcus faecium</i> , <i>Lactobacillus brevis</i> , <i>Lactobacillus acetotolerans</i> , <i>Lactococcus raffinolactis</i> , <i>Acetobacter aceti</i> , <i>Acetobacter lovaniensis</i> , <i>Acetobacter pasteurianus</i> , <i>Brettanomyces custersianus</i> , <i>Candida silvae</i> , <i>Issatchenkia orientalis</i> , <i>Saccharomyces cerevisiae</i>	16S and 26S rRNA genes	Ongol and Asano (2009)
Cassava for gari production	West Africa	<i>Lactobacillus plantarum</i> , <i>Lactobacillus fermentum</i> , <i>Lactobacillus pentosus</i> , <i>Lactobacillus acidophilus</i> , <i>Lactobacillus casei</i>	16S rRNA gene	Oguntoyinbo and Dodd (2010)
<i>Soy-based products</i>				
Chinese liquor	China	<i>Lactobacillus acetotolerans</i>	16S rRNA gene	Wang et al. (2008)
Daqus		<i>Saccharomycopsis fibuligera</i> , <i>Pichia anomala</i> , <i>Aspergillus oryzae</i> , <i>Absidia blakesleeana</i>	18S rRNA gene	Wang et al. (2011a)
Douchi		<i>Bacillus subtilis</i> , <i>Aspergillus oryzae</i>	16S and 18S rRNA genes	Chen et al. (2011a)
		<i>Lactococcus lactis</i> , <i>Staphylococcus lentus</i>	16S rRNA gene	Chen et al. (2011b)

Table 1 (continued)

Food products	Country	Microbial species identified	Target gene(s)	Reference
		<i>Bacillus subtilis</i> , <i>Bacillus amyloliquefaciens</i> , <i>Saccharomyces cerevisiae</i> , <i>Pichia farinosa</i>	16S and 26S rRNA genes	Chen et al. (2012)
Soy sauce	Japan	<i>Weissella cibaria</i> , <i>Staphylococcus gallinarum</i> , <i>Staphylococcus kloosii</i> , <i>Aspergillus oryzae</i> , <i>Zygosaccharomyces rouxii</i> , <i>Candida etchellsii</i> , <i>Candida versatilis</i>	16S and 26S rRNA genes	Tanaka et al. (2012)
Chungkookjang	Korea	<i>Pantoea agglomerans</i> , <i>Bacillus subtilis</i> , <i>Pantoea ananatis</i> , <i>Bacillus licheniformis</i>	16S rRNA gene	Hong et al. (2012)
Doenjang		<i>Leuconostoc mesenteroides</i> , <i>Tetragenococcus halophilus</i> , <i>Enterococcus faecium</i> , <i>Mucor plumbeus</i> , <i>Debaryomyces hansenii</i>	16S and 18S rRNA genes	Kim et al. (2009)
Japanese and Chinese fermented soybean pastes		<i>Tetragenococcus halophilus</i> , <i>Staphylococcus gallinarum</i> , <i>Aspergillus oryzae</i> , <i>Zygosaccharomyces rouxii</i>	16S and 18S rRNA genes	Kim et al. (2010a, 2010b)
Meju		<i>Enterococcus durans</i> , <i>Bacillus subtilis</i> , <i>Absidia corymbifera</i> , <i>Aspergillus</i> sp., <i>Candida rugosa</i>	16S and 18S rRNA genes	Lee et al. (2010)
Kochujang	South Korea	<i>Bacillus ehimensis</i> , <i>Bacillus megaterium</i> , <i>Bacillus pumilus</i> , <i>Bacillus subtilis</i> , <i>Bacillus licheniformis</i>	16S rRNA gene	Park et al. (2009)
Food spoilage				
Artisan-type cooked ham	Belgium	<i>Carnobacterium divergens</i> , <i>Brochothrix thermosphacta</i> , <i>Leuconostoc carnosum</i>	16S rRNA gene PCR-DGGE	Vasilopoulos et al. (2008)
Packaged sliced cooked meat		<i>Lactobacillus sakei</i> , <i>Lactobacillus fuchuensis</i> , <i>Lactobacillus curvatus</i> , <i>Carnobacterium divergens</i> , <i>Leuconostoc carnosum</i>	16S rRNA gene PCR-DGGE	Audenaert et al. (2010)
Raw milk	Canada	<i>Clostridium tyrobutyricum</i> , <i>Clostridium sporogenes</i> , <i>Clostridium disporicum</i>	16S rRNA gene PCR-DGGE	Julien et al. (2008)
Broiler Meat	China	<i>Staphylococcus</i> sp., <i>Pseudomonas</i> sp., <i>Carnobacterium</i> sp., <i>Weissella</i> sp.	16S rRNA gene PCR-DGGE	Zhang et al. (2012)
Chilled pork		<i>Pseudomonas</i> sp., <i>Lactobacillus</i> sp., <i>Brochothrix thermosphacta</i> , <i>Staphylococcus</i> sp., <i>Arthrobacter</i> sp., <i>Enterococcus</i> sp., <i>Moraxella</i> sp.	16S rRNA gene PCR-DGGE	Li et al. (2006)
Packaged pork		<i>Pseudomonas</i> sp., <i>Pseudomonas fluorescens</i> , <i>Brochothrix thermosphacta</i> , <i>Achromobacter xylosoxidans</i>	16S rRNA gene PCR-DGGE	Jiang et al. (2011)
Packed cooked ham		<i>Weissella viridescens</i> , <i>Leuconostoc mesenteroides</i>	16S rRNA gene PCR-DGGE	Han et al. (2011)
Prepared chicken products		<i>Weissella viridescens</i> , <i>Weissella minore</i> , <i>Acinetobacter</i> sp., <i>Carnobacterium</i> sp., <i>Pseudomonas</i> sp., <i>Brochothrix</i> sp., <i>Weissella</i> sp.	16S rRNA gene PCR-DGGE	Han et al. (2010) Liang et al. (2012)
Vacuum-packaged pork during chilled storage		<i>Carnobacterium</i> sp./divergens, <i>Lactobacillus sakei</i> , <i>Lactococcus</i> sp./piscium	16S rRNA gene PCR-DGGE	Jiang et al. (2010)
Beef chops	Italy	<i>Brochothrix thermosphacta</i> , <i>Pseudomonas</i> spp., <i>Carnobacterium divergens</i>	16S rRNA gene PCR-DGGE	Ercolini et al. (2011)
Beef stored in nisin activated packaging		<i>Pseudomonas</i> spp., <i>Carnobacterium</i> spp., <i>Carnobacterium divergens</i> , <i>Staphylococcus xylosus</i>	16S rRNA gene PCR-DGGE	Ercolini et al. (2010)
Cheese in late blowing spoilage		<i>Clostridium butyricum</i> , <i>Clostridium tyrobutyricum</i> , <i>Clostridium sporogenes</i>	16S rRNA gene PCR-DGGE	Cocolin et al. (2004a)
Fresh sausages		<i>Brochothrix thermosphacta</i> , <i>Lactobacillus sakei</i> , <i>Debaryomyces hansenii</i>	16S and 26S rRNA gene PCR-DGGE	Cocolin et al. (2004b)
Freshly cut beefsteaks		<i>Rahnella aquatilis</i> , <i>Pseudomonas</i> spp., <i>Carnobacterium divergens</i> , <i>Lactobacillus sakei</i>	16S rRNA gene PCR-DGGE	Ercolini et al. (2006)
Packaged Beef		<i>Lactobacillus sakei</i> , <i>Brochothrix thermosphacta</i> , <i>Leuconostoc</i> spp.	16S rRNA gene PCR-DGGE	Fontana et al. (2006)
Spoiled wines		<i>Brettanomyces bruxellensis</i> , <i>Saccharomyces cerevisiae</i> , <i>Brettanomyces anomalus</i>	26S rRNA gene PCR-DGGE	Cocolin et al. (2004c)
Wheat bread		<i>Bacillus subtilis</i> , <i>Bacillus licheniformis</i> , <i>Bacillus clausii</i> , <i>Bacillus firmus</i>	16S rRNA gene PCR-DGGE	Pepe et al. (2003)
Packaged beef	New Zealand	<i>Carnobacterium</i> spp., <i>Clostridium</i> spp.	16S rRNA gene PCR-DGGE	Brightwell et al. (2009)

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Table 1 (continued)

Food products	Country	Microbial species identified	Target gene(s)	Reference
Atlantic cod	Norway	<i>Photobacterium phosphoreum</i> , <i>Pseudomonas</i> spp., <i>Shewanella baltica</i> , <i>Shewanella putrefaciens</i>	16S rRNA gene PCR-DGGE	Hovda et al. (2007a)
Farmed atlantic cod		<i>Pseudomonas</i> spp., <i>Photobacterium</i> spp., <i>Shewanella putrefaciens</i>	16S rRNA gene PCR-DGGE	Hovda et al. (2007b)
Morcilla de Burgos	Spain	<i>Weissella viridescens</i> , <i>Leuconostoc</i> <i>mesenteroides</i> , <i>Weissella confusa</i> , <i>Gamma proteobacterium</i>	16S rRNA gene PCR-DGGE	Diez et al. (2008)
Pasteurized milk	United States	<i>Pseudomonas</i> sp., <i>Streptococcus</i> sp., <i>Buttiauxella</i> sp.	16S rRNA gene PCR-DGGE	He et al. (2009)

involved in the acidification process and in the proteolysis that results from their production of proteolytic enzymes, but may also play a role in the development of the organoleptic characteristics of cheese during the ripening period. Recently, it has been demonstrated that, during ripening, *L. lactis* may be involved in several pathways, such as carbohydrate and amino acids metabolisms, protein degradation and lipolysis (Desfossés-Foucault, 2012).

Apart from the detection of a metabolically active population, RNA targeted DGGE has recently shown another advantage, compared to DGGE, when analyzing DNA. In a study performed by Dolci et al. (2013) on the surface microbiota of Fontina, a smear cheese from the Aosta Valley region, North Italy, it was noticed that when subjected to an image analysis, the DGGE profiles obtained from the RNA extracted from the surface of the cheese, were clustering apart from the respective DNA samples. This result allowed the authors to speculate that RNA molecules may be a better target to describe the microbial ecology of complex microbial ecosystems, such as the rind of smear cheeses. This evidence could be due to the high number of ribosomes that metabolically active cells possess. In these circumstances, the PCR amplification should also be able to pick out microbial populations that are numerically low, but metabolically active, and should allow their specific signals to be detected in the DGGE gels. This outcome has also recently been described for table olive fermentation (Cocolin 2012, personal communication).

4.2. DGGE is a useful tool to assess product-specific microbial biodiversity

Food fermentations are microbial transformations in which a large number of microorganisms, belonging to different species and genera, compete to establish their supremacy. DGGE has been demonstrated to be able to follow the dynamic changes that occur during food fermentation and highlight dominant microbial populations. Its potential has been demonstrated by Cocolin et al. (2007) in a study in which an optimization of the DGGE procedures was carried out to study different sets of primers that are often used in DGGE analysis, denaturing gradients and electrophoretic conditions. When the optimized protocol was applied to fresh and ripened meat as well as dairy products, the ecological pressure exerted by certain microbial groups, namely LAB, during the fermentation process, became evident. The fresh produce was characterized by higher biodiversity, as observed from the complex patterns, while ripened products presented just a few bands, corresponding to the species that were able to dominate the microbial ecosystem. In the specific sector of meat fermentation, the main products that were investigated by DGGE were sausages from Italy, Argentina and Portugal (Table 1). LAB and coagulase negative cocci (CNC) are the main microbial groups responsible for fermentation and transformation, and this evidence was confirmed through an analysis of the DGGE profiles obtained in the above-mentioned studies. However, it should be noted that microbial competition occurs in the very early stages of

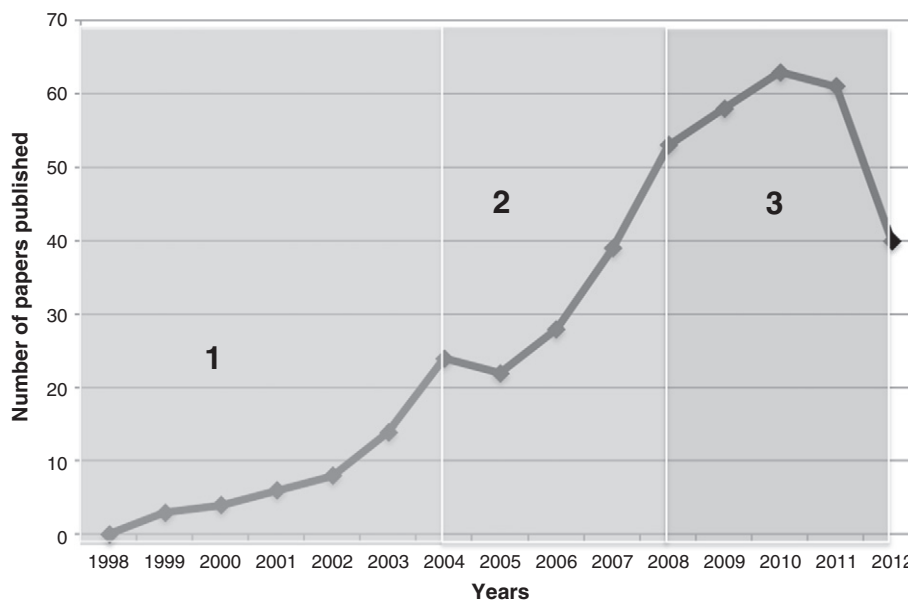


Fig. 1. Trend in the publication of papers using DGGE as culture-independent method. The data were obtained from the Scopus database (www.scopus.com) in December 2012, using the keywords "DGGE and food".

fermentation, since, as demonstrated by Cocolin et al. (2001a), already at the third day, the signals of *Lb. sakei* and *Lb. curvatus*, the main species involved in sausage fermentation, became predominant. In studies conducted on fermented sausage ecology it has often been highlighted how DGGE profiles could be used to differentiate products from different geographic regions and production plants. This result was obtained for Argentinian sausages (Fontana et al., 2005a), and also for sausages produced in North–East Italy (Rantsiou et al., 2005). More specifically, in the latter study, the microbial changes that occurred during fermentations of the three products, with a ripening period of 28, 45 and 120 days, respectively, were investigated. Through a cluster analysis of the DGGE profiles, it was demonstrated that sausages at the beginning of the fermentation (from 3 to 7 days) present DGGE patterns that do not show any similarity with other samples, while from day 10 onwards the different types of sausages started to group together in a product-specific clustering manner.

4.3. DGGE analysis highlights hidden populations

As already mentioned above, one of the main criticisms that is currently directed towards traditional microbiological methods concerns the impossibility of detecting microorganisms in low numbers in complex ecosystems with dominant populations. DGGE has partially overcome this limitation, having a limit of detection of about 10^3 cfu/ml or g (Cocolin et al., 2001a, 2001b).

In several food fermentation sectors, the DGGE technique has been able to highlight populations that may have an important impact on the final characteristics of the product, but which were not well described by means of traditional methods. An interesting example, in this context, is the application of DGGE to wine fermentation. The ecology of wine yeasts has been the focus of a large amount of literature, starting from the 80s, which has indicated *Saccharomyces cerevisiae* as the main responsible for alcoholic fermentation (Fleet and Heard, 1993), which is able to dominate over other yeast species, collectively called non-*Saccharomyces*. This last group is usually of concern in the wine making sector, since it contains yeasts that are detrimental to the quality of wine. There is scientific consensus on the capability of *S. cerevisiae* to take over the wine fermentation process. However, the role of non-*Saccharomyces* is still under debate. The use of DGGE during wine fermentation has often demonstrated an active participation of non-*Saccharomyces* yeasts in the fermentation process, and their persistence is longer than what was previously believed. Ecological studies on a sweet wine produced in California (USA), called Dolce, produced from botrytized grapes, highlighted multiple bands in DGGE profiles that, after sequencing, were identified as putative *Candida stellata*. These bands were present from the very beginning of the fermentation and remained stable throughout the fermentation process (Cocolin et al., 2001b). In another study conducted the following year (Mills et al., 2002), the active participation of this *Candida* sp. was confirmed and it was demonstrated, by RNA dot blot analysis, that viable populations of at least 10^6 cfu/ml were present at the end of the fermentation. This species was classified as a new member of the *Candida* genus and it was given the name *C. zemlinina* by Sipiczki in 2003 and it is nowadays considered as a possible fermentation partner of *S. cerevisiae* in mixed fermentations to reduce the acetic acid content in sweet wines (Rantsiou et al., 2012). The concept of mixed fermentations is not new in wine making, however the results obtained by means of culture-independent methods support the idea of exploiting some positive contributions of non-*Saccharomyces* yeasts to obtain wines with more complex organoleptic profiles.

5. Conclusions and future perspective

The application of culture-independent methods to food microbiology is relatively new and the last 15 years have been particularly exciting for those working in the field of microbial food ecology. For the first time

in the history of microbiological food examination, scientists have had methods at hand that do not rely on cultivation, and which are able to study microbial populations that had not been detected previously on synthetic media, because they had been overgrown with the dominant microbiota, or because they had been in VBNC states. DGGE, as the most representative technique in this context, has been used extensively and a large amount of literature has been written for different types of fermented food and for food spoilage processes.

The last couple of years have seen the introduction of new methodologies in microbial food ecology and these are expected to increase in number over the next few years. Next generation sequencing (NGS) has a great advantage over DGGE. In the latter case, only intense and well separated bands can be sequenced in the profiles, and as a consequence, only a partial fraction of the microbiota in that specific food sample can be assessed and identified, but with NGS a massive quantity of sequences are generated from a single sequencing run, and the analysis of this run offers the possibility of obtaining a large amount of information in a relatively short time. When applied to microbial ecology studies, NGS makes it possible to determine how many reads of different operational taxonomic units (OTUs) occur in a template and therefore to have an estimation of the percent of occurrence of different OTUs in a specific ecosystem. This modern molecular approach has been used in the field of applied food microbiology to study the ecology of pearl millet slurries (Humboldt and Guyot, 2009), the microbiota of different kinds of cheese (Alegria et al., 2012; Quigley et al., 2012; Ercolini et al., 2012; Masoud et al., 2011), the microbial diversity of Brazilian kefir grains (Leite et al., 2012) and the fermentation dynamics of different Asian foods (Nam et al., 2012a,b; Park et al., 2012). However, NGS offers the even more interesting possibility of studying the occurrence and abundance of microbial genes in a given ecosystem and of establishing how these genes are expressed. Metagenomic and metatranscriptomic studies represent the future for the study of the microbial ecology of food. In a few years, detailed data on the ecology of microbial transformations in food will become available, allowing scientists to fully comprehend the role and impact of specific microorganisms in defined food sectors. It is expected that new information will become available which will be used to improve food quality and safety.

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