Chapter 4
Molecular Methods for Identification of Microorganisms in Traditional Meat Products

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Introduction

Traditional fermentations are those that have been used for centuries and even pre-date written historical records. Fermentation processes have been developed to upgrade plant and animal materials, to yield a more acceptable food, to add flavor, to prevent the growth of pathogenic and spoilage microorganisms, and to preserve food without refrigeration (Hesseltine & Wang, 1980). Among fermented foods, sausages are the meat products with a longer history and tradition. It is often assumed that sausages were invented by the Sumerians, in what is Iraq today, around 3000 BC. Chinese sausage *làcháng*, which consisted of goat and lamb meat, was first mentioned in 589 BC. Homer, the poet of The Ancient Greece, mentioned a kind of blood sausage in the Odyssey (book 20, verse 25), and Epicharmus (ca. 550 BC–ca. 460 BC) wrote a comedy entitled "*The Sausage*". Evidence suggests that sausages were already popular both among the ancient Greeks and Romans (Lücke, 1974).

Today, there is a great variety and diversity of fermented sausages in the market world-wide, as a consequence of different formulations used in their production. The ingredients, such as raw materials and spices, together with manufacturing practices and fermentation techniques are the main factors that lead to the production of fermented sausages with specific organoleptic profiles and physico-chemical characteristics. However, a general distinction can be made between fermented products that are produced in the Northern countries and the ones manufactured in areas with temperate climate, mainly the Mediterranean countries. Northern products have a pH below 5, while Mediterranean products have a pH of 5.3–6.2 and are highly desiccated (Talon, Leroy, & Lebert, 2007).

The fermentation of sausages is a microbial process that has been investigated since 1960 (Lerche & Reuter, 1960; Lücke, 1974; Reuter, 1972), and these studies highlighted that the main microorganisms responsible for the transformation are lactic acid bacteria (LAB, *Lactobacillus* spp.) and coagulase-negative cocci

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(CNC, *Staphylococcus* and *Kocuria* spp.). Moreover, in some fermented sausages, especially those produced in France, Italy, and Spain, the characteristics of the final products are influenced by the activity of molds and yeasts that are developing on the surface of the product (Lücke, 2000).

Microbial dynamics during fermentation have a tremendous impact on the sensory properties of the product. Sausages with lower pH are characterized by a short ripening time and have more lactobacilli from the early stages of fermentation. An acid flavor predominates in the product and commercialization takes place after less than two weeks of ripening. Long ripening times allow for more diverse microbial activities. LAB are mainly responsible for acidification. They are able to reduce the pH of the sausages by production of lactic acid from carbohydrates (Hammes, Bantleon, & Min, 1990; Hammes & Knauf, 1994). Moreover, they influence the sensory characteristics of the fermented sausages by the production of small amounts of acetic acid, ethanol, acetoin, pyruvic acid, and carbon dioxide (Bacus, 1986; Demeyer, 1982), and they are able to initiate the production of aromatic substances thanks to the proteolytic activity of muscle sarcoplasmatic proteins (Fadda et al., 1999a, 1999b). Apart from LAB, also CNC and yeasts contribute to the final characteristics, due to their capability to produce higher levels of volatile compounds with low sensory thresholds (Demeyer, Verplaetse, & Gistelink, 1986). CNC also have a fundamental role in the development and stability of the red color through the formation of nitrosomyoglobin by nitrate reductase activity, possibly involved in the limitation of the lipid oxidation, as well (Talon, Walter, Chartier, Barriere, & Montel, 1999). Furthermore, CNC are able to produce proteolytic and lipolytic enzymes responsible for the release of low molecular weight compounds, such as peptides, amino acids, aldehydes, amines, and free fatty acids, that are influencing the aromatic profile of the final product (Demeyer et al., 1986; Schleifer, 1986).

In the last 10 years, the approach to study microbial biodiversity has changed dramatically. With the advancement of molecular biology and the invention of the polymerase chain reaction (PCR), a new range of techniques were developed that can help in the understanding of the microbial complexity in natural ecosystems. As a consequence, the traditional microbiological techniques, based on plating, isolation, and biochemical identification, are now supported by new methods that rely on the analysis of the nucleic acids for detection, identification, and characterization of the microorganisms. In this context, several groups of food scientists started to apply molecular methods to various food fermentations. The aim of this chapter is to report on the molecular methods used so far to identify, characterize, and profile microbial diversity during the fermentation of sausages.

Approaches Used to Study the Microbial Diversity

The scientific community recognizes that the use of methods that are relying on the cultivation of the microorganisms (culture-dependent techniques), do not properly profile the microbial diversity present in a specific ecosystem (Hugenholtz, Goebel,

& Pace, 1998). As a matter of fact, populations that are numerically limited or microorganisms that are stressed or in a sub-lethal state cannot be recovered, thereby they are eliminated from consideration. Moreover, viable but not culturable (VNC) cells that are not able to form colonies on agar plates but possess metabolic activity will not be picked up by culture-dependent methods. Methods that do not depend on cultivation (culture-independent techniques) have attracted the attention of many scientists in different domains of investigation, spanning from the environmental microbiology to food fermentations. Generally, they are based on the analysis of the DNA and RNA that is extracted from the sample directly without any kind of cultivation. The nucleic acids are then amplified by PCR and subjected either to cloning and sequencing or to profiling techniques, such as denaturing gradient gel electrophoresis (DGGE) or temperature gradient gel electrophoresis (TGGE), or single strand conformation polymorphism (SSCP). An alternative, culture-independent approach that does not rely on extraction of nucleic acids from the sample matrix is the fluorescence in situ hybridization (FISH). In this case, not only it is possible to identify the microorganisms by using specific probes, but it also permits the localization of species within the sample being investigated. In Fig. 4.1, the culture-dependent and independent methods are summarized.

Finally, the extensive development of molecular methods resulted in new tools that could be used for molecular characterization of the strains isolated by culture-dependent methods. Randomly amplified polymorphic DNA (RAPD)-PCR,

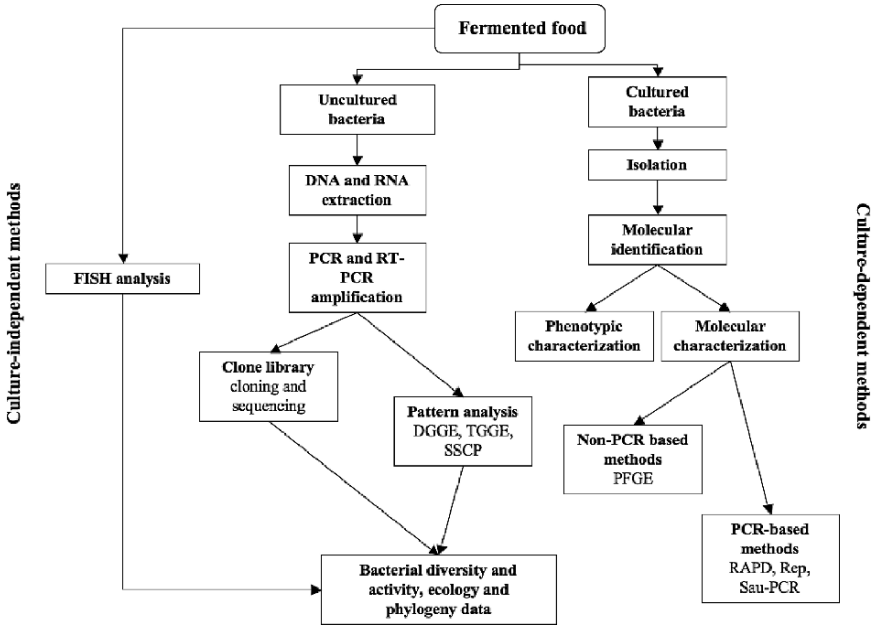

Fig. 4.1 Culture-dependent and -independent methods used to study the microbial ecology of fermented foods (Modified from Pontes et al., 2007).

repetitive bacterial DNA elements (Rep)-PCR, and enterobacterial repetitive intergenic consensus (ERIC)-PCR are common practices in almost all the laboratories that deal with microbial ecology and diversity. At present, it is widely accepted that different methods for the identification and characterization of microbial species in a specific ecosystem are necessary. The combination of different methods to study the microbial ecology of a system is defined as a "polyphasic approach" (Pontes, Lima-Bitterncourt, Chartone-Souza, & Amaral Nascimiento, 2007).

Molecular Methods Used to Investigate the Diversity of Microorganisms in Fermented Sausages

As mentioned above, an important distinction that has to be made is between culture-dependent and independent methods. While the culture-dependent methods are commonly used to identify and molecularly characterize microbial isolates, the culture-independent methods are used to profile directly the microbial populations during fermentation of sausages (Rantsiou & Cocolin, 2006).

Culture-Dependent Techniques

Strain Identification

The identification of strains isolated from sausage fermentation is most commonly achieved by targeting the ribosomal RNA operon (rRNA). It possesses some requisites and attributes that are very important for microbial identification. Apart from being present in all microbial species, it is characterized by the presence of conserved and variable regions, that become very important for differentiation purposes, and it functions as an evolutionary clock, giving the possibility to draw conclusions regarding evolution and phylogeny. In the case of the bacteria, the 16S rRNA gene is the most used target for molecular applications (Collins et al., 1991), while the D1-D2 loop of the 26S RNA gene became a generally accepted target for yeast identification (Kurtzman & Robnett, 1997). The process of identification of microbial strains by targeting the rRNA usually consists of a first step in which the specific gene (16S or 26S rRNA), or portion of it, is amplified by PCR that subsequently is subjected to DNA sequencing. By alignment of the sequence in the GeneBank, using available software programs online (Blast, Altschul et al., 1997), it is possible to achieve the identification. This experimental tactic is not applicable for ecology studies because of the high number of isolates; it is not feasible and economically convenient to sequence hundreds of strains. For this reason, researchers developed species-specific PCR and ribosomal RNA probes as fastest ways to obtain species identification. Ribosomal RNA probes (Nissen & Dainty, 1995), species-specific PCR primers (Berthier & Ehrlich, 1998; Blaiotta, Pennacchia, Parente, & Villani, 2003; Morot-Bizot, Talon, & Leroy-Setrin, 2003; Rossi, Tofalo, Torriani, & Suzzi, 2001), and multiplex PCR (Corbiere Morot-Bizot, Talon, & Leroy, 2004)

have been used in the field of fermented sausages for the identification of LAB and CNC isolated during fermentation.

Alternatively, the PCR-sequencing methodology is coupled with techniques that are able to differentiate strains based on fingerprinting profiles, allowing grouping of the isolates and reducing the number of strains requiring sequencing. The methods used to group strains and reduce the number of isolates to sequence are once more based on an amplification step. The differences in the strains to identify can be detected either by exploiting primers that are annealing in various regions of the genome of the strain to identify, thereby producing a band pattern that is representative for a species, or by detecting differences in the DNA sequences specifically amplified by PCR. The first group of methods is mainly represented by RAPD-PCR and Rep-PCR. RAPD-PCR is characterized by an amplification process, using a single random primer that will anneal in several portions of the genomic DNA and give amplification products when the forward and reverse annealing sites are allowing extension by the DNA polymerase (Caetono-Anollés, 1993; Power, 1996). The main drawback of this technique is its low reproducibility. The annealing step takes place at very low temperature (37–40°C), and for this reason, the same strain subjected to RAPD-PCR in different moments can produce different profiles. While it is possible to reach a good intra-laboratory reproducibility, if all the experimental steps are optimized and standardized, it is practically impossible to be able to compare the results obtained in different laboratories. Concerning Rep-PCR, the primers used are specific to the repetitive elements spread around the bacterial genomes (Gilson, Clement, Brutlag, & Hofnung, 1984), and for this reason the profiles obtained are highly specific for a species and they are highly reproducible, as well. RAPD-PCR and Rep-PCR are methods that were first applied for identification purposes (Andrighetto, Zampese, & Lombardi, 2001; Berthier & Ehrlich, 1999; Gevers, Huys, & Swings, 2001; Rebecchi, Crivori, Sarra, & Cocconcelli, 1998), and only recently they have been used for molecular characterization of LAB and CNC (Cocolin et al., 2004; Comi et al., 2005; Iacumin, Comi, Cantoni, & Cocolin, 2006a; Rantsiou, Drosinos, Gialitaki, Urso, et al., 2005, Rantsiou, Iacumin, Cantoni, Comi, & Cocolin, 2005; Rantsiou, Drosinos, Gialitaki, Metaxopoulos, et al., 2006; Urso, Comi, & Cocolin, 2006), as well as yeasts (Cocolin, Urso, Rantsiou, Cantoni, & Comi, 2006a) isolated from fermented sausages.

The techniques that are allowing strain grouping based on differences in a DNA sequence and that which have been applied in the field of fermented sausages are the restriction fragment length polymorphism (RFLP) analysis of the 16S rRNA gene (Lee et al., 2004; Sanz, Hernandez, Ferrus, & Hernandez, 1998) and the denaturing/temperature gradient gel electrophoresis (D/TGGE) (Blaiotta, Pennacchia, Ercolini, Moschetti, & Villani, 2003; Cocolin, Manzano, Cantoni, & Comi, 2000; Cocolin, Manzano, Aggio, Cantoni, & Comi, 2001; Cocolin, Manzano, Cantoni, & Comi, 2001; Ercolini, Moschetti, Blaiotta, & Coppola, 2001a). With the RFLP approach, the differences in the DNA sequence are identified by using a restriction endonuclease that is cutting the DNA in specific restriction sites, giving a specific pattern for a given species. The D/TGGE methods are detecting differences in the sequences by analyzing their denaturation behavior. More specifically, as the

DNA molecule encounters an appropriate denaturant concentration, a sequence-dependent partial denaturation of the double strand occurs. The change in the DNA conformation determines a reduction of the migration rate of the molecule, and thereby DNA with different nucleotide sequences will show differential electrophoretic patterns. This approach was first used in environmental ecosystems (Muyzer, de Waal, & Uitterlinden, 1993), and at the beginning of the 21st century an impressive number of papers applying D/TGGE in the field of food microbiology have been published (for review see Ercolini, 2004).

Strain Characterization

Strains isolated during the fermentation of sausages can be also subjected to molecular characterization, in order to understand the dynamics and diversity within the same species. Usually, these approaches are applied to isolates that have previously been identified with other molecular techniques (see above) and intra-species differences are highlighted. Only the strains belonging to the same species can be subjected to this kind of characterization. Also in this case, the methods that are used are divided into methods that are exploiting a PCR amplification and non-PCR methods. In the case of PCR-based methods, once more the RAPD-PCR and Rep-PCR are well-established techniques to look into the molecular diversity within selected species of bacteria or yeasts. RAPD-PCR has been applied to examine the diversity of *Lactobacillus sakei* in naturally fermented Italian sausages, in order to understand the RAPD-types that were able to take over the fermentation (Urso et al., 2006), and to differentiate *Debaryomyces hansenii* strains from the sausages fermented at low temperature (Cocolin et al., 2006a). Rep-PCR was used in order to characterize *Staphylococcus xylosus* strains isolated from three fermentation processes in Northern Italy (Iacumin et al., 2006a). In addition, in this study, a new technique, named Sau-PCR, developed for the characterization of dairy-related microorganims (Corich, Mattiazzi, Soldati, Carraro, & Giacomini, 2005), was applied for the first time to differentiate the strains of meat origin. This method is based on DNA digestion with the restriction endonuclease *Sau*3A, followed by amplification, using one primer that contains the sequence of the restriction site of the enzyme, and thereby is able to anneal to the ends of the fragments obtained. The result is a pattern of amplified bands that represents the restriction fragments obtained by digestion with the specific endonuclease. Using the same rational, several enzymes can be used in order to obtain a more precise molecular characterization.

Regarding the molecular methods that do not exploit an amplification step, the only one used in the field of fermented meats is represented by the pulsed-field gel electrophoresis (PFGE). Currently, this technique is considered the “gold standard” for the characterization of strains, since it is very precise, reproducible, and reliable. In the field of food borne pathogens, a database of PFGE profiles was created (PulseNet, http://www.cdc.gov/pulsenet/), thereby allowing epidemiologic analysis of food borne disease outbreaks. This is possible because the results obtained by PFGE are comparable between different laboratories thanks to the reproducibility

of the method. PFGE is an electrophoretic method that allows separation of the chromosomes for the yeasts and of macro-restriction fragments obtained from the genomic DNA for bacteria (Tenover et al., 1995). The first step is the extraction of the DNA from the microorganism that is embedded in agarose gel and subjected to lysis treatments, in order to digest the cell wall. Once the DNA has been freed, it can be subjected directly to PFGE or it is digested with a restriction endonuclease that generally has the characteristic to be rare-cutting. In this way, a relatively small set of restriction fragments is obtained that are simply resolved in an agarose gel. The electrophoresis is carried out in specific chambers, in which the electric field is changing orientation. In this way, the DNA molecules are separated more efficiently than in conventional electrophoresis. The result obtained is a band pattern that is specific to the strain that was subjected to the PFGE analysis. Despite the fact that PFGE has been applied several times to characterize strains of dairy-origin, it was applied rarely to LAB and CNC isolated from fermented sausages. PFGE was used to monitor *S. xylosus* starter cultures, both in Southern Italy (Di Maria, Basso, Santoro, Grazia, & Coppola, 2002) and in two French processing plants (Corbiere Morot-Bizot, Leroy, & Talon, 2007), and to study the diversity of *Lactobacillus* strains isolated from fermented sausages to be used as potential probiotics (Pennacchia, Vaughan, & Villani, 2006).

Culture-Independent Techniques

As mentioned above, the culture-independent methods are able to profile the microbial populations in complex microbial ecosystems without any cultivation. The strategy based on which these methods are working is the analysis of the nucleic acids extracted directly from the matrix. Once the DNA and RNA are available, they can be subjected to several types of analysis that can be preceded by a PCR step or not. The culture-independent method that has been more extensively applied to sausage fermentation is the DGGE. As already reported above, the method is able to differentiate DNA molecules based on their denaturation behaviors. When the method is used for microbial population profiling, the PCR is carried out with universal primers, able to prime amplification for all the microbes present in the sample. After this step, a complex mixture of DNA molecules will be obtained, that can be differentiated if separated in gels with denaturant gradients. Every single band that is visible in D/TGGE gels represents a component of the micro-biota. The more bands are visible, the more complex is the ecosystem. By using these methods, it is possible not only to profile the microbial populations, but also to follow their dynamics during time. However, these methods are not quantitative. DGGE analysis has been applied mainly to Italian fermented sausages (Cocolin, Manzano, Cantoni, et al., 2001; Rantsiou, Urso, et al., 2005; Silvestri et al., 2007; Villani et al., 2007), but studies on the fermentation dynamics of Argentinean sausages are available as well (Fontana, Cocconcelli, & Vignolo, 2005; Fontana, Vignolo, & Cocconcelli, 2005).

The use of species-specific primers in the PCR amplification, analyzing the DNA extracted directly from the sample, can be considered to be another culture-independent method. This approach was so far used to identify LAB and CNC species in Spanish fermented sausages (Aymerich, Martin, Garriga, & Hugas, 2003). Moreover, with the last technological improvements that allowed the PCR to become a quantitative method, direct enumeration of technologically important species during fermentation of sausages can be achieved. This was described for the first time by Martin, Jofré, Garriga, Pla, and Aymerich, (2006), who optimized a quantitative PCR (qPCR) protocol for the rapid quantitative detection of *Lb. sakei* in fermented sausages.

Lastly, a promising culture-independent method that, unfortunately, has not yet been efficiently exploited to study the microbial diversity in fermented sausages, is the FISH. In this technique a set of specific probes are used to target different microorganisms directly in the sample. The probes are labeled with different fluorophores, thereby allowing the detection of several species simultaneously. Since the probes are generally designed on the ribosomal RNA, only alive cells are detected by FISH (Bottari, Ercolini, Gatti, & Neviani, 2006). One of the most fascinating features of FISH is the possibility to localize the microorganisms directly into the food matrix. This has been applied to dairy products (Ercolini, Hill, & Dodd, 2003), but never to fermented sausages. The only application of FISH to fermented and fresh sausages to profile the microbial populations in food products has been described by Cocolin et al. (2007).

The Microbial Ecology of Fermented Sausages as Determined by Culture-Dependent Methods

The experimental approach used by researchers in order to describe the microbial ecology of fermented sausages, using culture-dependent methods, is summarized in Fig. 4.2, and the list of studies published using these techniques for the identification and characterization of isolates are reported in Table 4.1. After homogenization, the sample is analyzed by plating onto a specific media to allow the selective growth of the microorganisms of interest. After incubation, counts and random isolations are performed. The isolates are subsequently subjected to molecular identification and characterization.

Molecular Methods for the Identification of Isolated Strains

In the nineties, molecular techniques for the identification of microorganisms isolated from fermented meat products, started to place side by side or to substitute biochemical assays. Although the two different approaches, in most of the cases, arrived at similar results, molecular techniques immediately showed a higher level of reproducibility, automatism, and fastness.

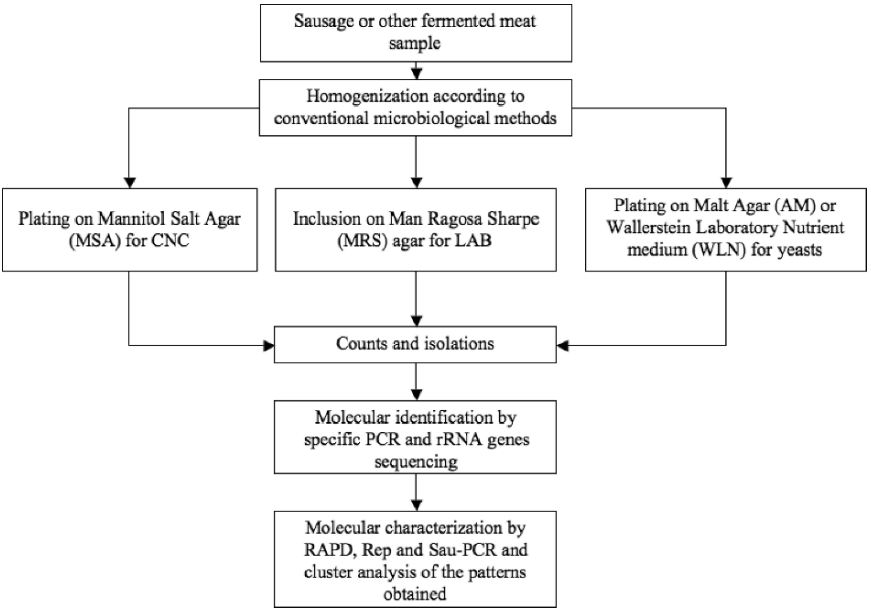

Fig. 4.2 Flowchart describing the application of culture-dependent methods for the identification and characterization of microbial species isolated from fermented sausages.

Specific Hybridization Probes

Although, in later papers, 16S rRNA became the gene more often targeted for bacterial identification (Aymerich et al., 2006; Blaiotta et al., 2004; Gory, Millet, Godon, & Montel, 1999; Rantsiou, Drosinos, Gialitaki, Metaxopoulos, et al., 2006), in 1991, Hertel et al. designed specific probes based on differences in the vicinity of the 5'-terminus of the 23S rRNA gene, to identify the meat lactobacilli *Lb. curvatus*, *Lb. sakei*, and *Lb. pentosus*. The specificity of the probes was checked by dot blot and colony hybridization. Oligonucleotide probes hybridized only with the rRNA gene targets of strains belonging to the corresponding species *Lb. curvatus* and *Lb. sakei*, closely related genetically but not easy to differentiate phenotypically. On the contrary, the probe directed towards the 23S rRNA gene of *Lb. pentosus* reacted positively with *Lb. plantarum*, too. These probes, however, were never used to identify isolates from fermented sausages.

Species-Specific PCR

A large quantity of papers, regarding the design of PCR species-specific primers, for the rapid and reliable identification of closely related bacterial species, were published from 1998, when Berthier and Ehrlich designed primers complementary to lactobacilli species-specific sequences in the 16S/23S rRNA gene spacer region. They were able to distinguish strains belonging to two groups of closely related

Table 4.1 Culture-dependent methods used for the identification and characterization of strains isolated from fermented sausages

Method	Type of analysis	Target group	Type of product	Type of study	Reference
Species-specific PCR	Identification	CNC	Traditional Slovak fermented sausages	Final product	Simonova et al., 2006
		LAB	Traditional French dry sausages	Throughout the fermentation	Ammor et al., 2005
Species-specific PCR and sequencing of 16S rRNA gene	Identification	LAB	Low acid Spanish fermented sausages	Final product	Aymerich et al., 2006
			Fermented Greek sausages		Rantsiou, Drosinos, Gialitaki, Metaxopoulos, et al., 2006
Multiplex PCR	Identification	CNC	Traditional French fermented dry sausages	Throughout the fermentation	Corbiere Morot-Bizot et al., 2006
PCR-TGGE	Identification	LAB	Naturally fermented Italian sausages	Final product	Cocolin, Manzano, et al., 2000
PCR-DGGE and sequencing of 16S rRNA gene	Identification	CNC	Naturally fermented Italian sausages	Throughout the fermentation	Cocolin, Manzano, Aggio, et al., 2001
		LAB			Cocolin, Manzano, Cantoni, et al., 2001; Comi et al., 2005
PCR-DGGE, sequencing of 16S rRNA gene and species-specific PCR	Identification	LAB	Naturally fermented sausages from Greece, Hungary and Italy	Throughout the fermentation	Rantsiou, Drosinos, Gialitaki, Urso, et al., 2005
			Naturally fermented Italian sausages		Urso et al., 2006
		CNC	Naturally fermented Italian sausages		Iacumin et al., 2006b

Table 4.1 (continued)

Method	Type of analysis	Target group	Type of product	Type of study	Reference
PCR-DGGE and sequencing of 26S rRNA gene	Identification	Yeasts	Naturally fermented Italian sausages	Throughout the fermentation	Cocolin et al., 2006a
ISR-PCR and DGGE	Identification	CNC	Traditional Southern Italian fermented sausages	Final product	Blaiotta, Pennacchia, Ercolini, et al., 2003
ISR-PCR	Identification	CNC	Artisanal Italian dry sausages	Throughout the fermentation	Rossi et al., 2001
ISR-PCR and species-specific PCR	Identification	CNC	Slightly fermented Spanish sausages	Final product	Martin, Garriga, et al., 2006
RFLP	Characterization	LAB	Dry-cured sausages	Final product	Sanz et al., 1998
	Identification	LAB and CNC	Italian dry sausages	Final product	Rebecchi et al., 1998
	Identification and characterization	LAB	Traditional fermented sausages	Final product	Andrighetto et al., 2001
RAPD-PCR	Characterization	LAB CNC *Bacillus* sp. Yeasts	Typical Southern Italian sausages (Salame di Senise)	Manufacturing and ripening	Baruzzi et al., 2006
		CNC	Artisanal Italian dry sausages		Rossi et al., 2001
		LAB CNC	Artisanal Argentine fermented dry sausages		Fontana, Cocconcelli, & Vignolo, 2005
		Yeasts	Fermented Italian sausages	Throughout the fermentation	Cocolin et al., 2006a
		LAB	Naturally fermented Italian sausages		Comi et al., 2005

Table 4.1 (continued)

Method	Type of analysis	Target group	Type of product	Type of study	Reference
RAPD-PCR	Characterization	LAB	Naturally fermented Italian sausages	Throughout the fermentation	Urso et al., 2006
			Naturally fermented sausages from Greece, Hungary and Italy		Rantsiou, Drosinos, Gialitaki, Urso, et al., 2005
			Fermented Greek sausages	Final product	Rantsiou, Drosinos, Gialitaki, Metaxopoulos, et al., 2006
RAPD-PCR and plasmid profiling	Characterization	LAB	Low acid Spanish fermented sausages	Final product	Aymerich et al., 2006
		CNC	Slightly fermented Spanish sausages		Martin, Garriga, et al., 2006
RAPD-PCR and rep-PCR	Characterization	LAB	Traditional Italian fresh sausages	Throughout the fermentation	Cocolin et al., 2004
RAPD-PCR, Sau-PCR and rep-PCR	Characterization	CNC	Naturally fermented Italian sausages	Throughout the fermentation	Iacumin et al., 2006a
rep-PCR	Characterization	CNC	Traditional Italian fresh sausages	Throughout the fermentation	Rantsiou, Iacumin, et al., 2005
PFGE and RAPD-PCR	Characterization	CNC	Fermented Italian sausages (Soppressata)	Throughout the fermentation	Di Maria et al., 2002
PFGE and multiplex PCR	Characterization and identification	LAB	Traditional Italian dry fermented sausages	Final product	Pennacchia et al., 2006
PFGE	Characterization	CNC	Traditional French dry fermented sausages	Throughout the fermentation	Corbiere Morot-Bizot et al., 2006

Lactobacillus species: one composed of *Lb. curvatus*, *Lb. graminis*, and *Lb. sakei* and the other of *Lb. paraplantarum*, *Lb. pentosus*, and *Lb. plantarum*. The next year the same authors (Berthier & Ehrlich, 1999) developed pairs of PCR primers that could be used to specifically detect *Lb. curvatus* and *Lb. sakei*. They evaluated the phenotypic and genotypic diversity among 165 isolates, previously assigned to *Lb. curvatus* and *Lb. sakei* species, by comparing the results of biochemical assays and RAPD data. This allowed them to consider the presence of species-specific RAPD bands that were cloned and sequenced. On the basis of differences in these sequences, species-specific primers were designed. Likewise, a pair of primers specific for *S. xylosus* were designed by Morot-Bizot et al. (2003) on a RAPD product of 539 bp, shared by *S. xylosus* strains from international collections. The validity of the method was confirmed by comparing the results obtained with the designed primers and by PFGE.

Since among the CNC species, *S. xylosus*, *S. equorum*, and *S. carnosus* are frequently involved in the fermentation of several typologies of meat products, a number of papers regarding the design of primers specific for these microorganisms were published. Blaiotta, Pennacchia, Parente, et al. (2003) used xylulokinase and a 60 kDa heat-shock protein coding genes as targets for the design of *S. xylosus* specific primers. They successfully evaluated the specificity of two sets of primers on 27 references strains of the Deutsche Sammlung von Mikroorganismen and Zellkulturen GmbH (DSM) (Germany) collection. Later on, Blaiotta, Ercolini, Mauriello, Salzano, & Villani, (2004) developed primers specific for *S. equorum* that targeted the manganese-dependent superoxide dismutase (*sodA*) gene and were successful in differentiating *S. xylosus* and *S. equorum* that are otherwise difficult to separate by biochemical or physiological traits. The reliability of the method was tested on a total of 112 strains, representing 26 different species of the genus *Staphylococcus*, 3 species of the genus *Kocuria*, and different strains of the *Macrococcus caseolyticus* species. Similarly, Blaiotta, Casaburi, and Villani (2005) designed two different sets of primers, targeting the *sodA* genes of *S. carnosus* and *S. simulans* and were able to differentiate the two species.

Simonova et al. (2006) employed primers previously described by other authors (Aymerich et al., 2003) to detect *S. xylosus* and *S. carnosus* strains in different types of Slovak traditional sausages, for the selection of potential starter cultures to be used in sausage processing. As in many Italian and Spanish fermented meat products (Blaiotta et al., 2004; Coppola, Iorizzo, Saotta, Sorrentino, & Grazia, 1997; Garcia-Varona, Santos, Jaime, & Rovira, 2000), *S. xylosus* was the dominating CNC (63.6%), while *S. carnosus* showed 10.7% frequency.

By using species-specific primers, Ammor et al. (2005) carried out a survey on LAB isolated in a small-scale facility producing traditional dry sausage in France. While *Enterococcus faecium*, *Vagococcus carniphilus*, and *Lactococcus garvieae* were the predominant species isolated from processing equipment and raw material, *Lb. sakei* became the predominant species during the fermentation of the product. *Lb. sakei* strains prevailed (43.3%) also among homo-fermentative mesophilic rods found by Greco, Mazzette, De Santis, Corona, & Cosseddu (2005) in the production and ripening of a typical dry fermented sausage from Sardinia (Italy). The authors used

species-specific primers to detect also *Lb. plantarum* and *Lb. curvatus* strains, which were isolated from the product in the percentages of 16.6 and 13.3%, respectively.

The biodiversity of lactobacilli was also evaluated in low acid Spanish fermented sausages and in naturally fermented Greek sausages, produced in three different processing plants, by Aymerich et al. (2006) and Rantsiou, Drosinos, Gialitaki, Metaxopoulos, et al. (2006), respectively. In both cases, isolates that could not be identified by species-specific PCR were identified by the partial sequencing of the 16S rRNA gene that allowed overcoming the limit of species-specific PCR method. In fact, in microbial ecology studies, it is unlikely to reach the identification of all isolates by species-specific PCR, because of the lack of primers or the presence of unexpected species. *Lb. sakei* was the predominant species (74%) in the Spanish fermented sausages (Aymerich et al., 2006), followed by *Lb. curvatus* (21.2%) and *Leuconostoc mesenteroides* (4.8%). The results obtained from Rantsiou, Drosinos, Gialitaki, Metaxopoulos, et al. (2006) highlighted that the main populations involved in the fermentations analyzed, belonged to the species *Lb. sakei*, *Lb. plantarum*, and *Lb. curvatus*, differently predominating depending on the plants considered.

Corbiere Morot-Bizot et al. (2004) developed a multiplex PCR for the identification of bacteria belonging to the *Staphylococcus* genus and in particular to the species *S. xylosus*, *S. saprophyticus*, *S. epidermidis*, and *S. aureus*. Later, the same authors (Corbiere Morot-Bizot et al., 2007) used this approach to investigate the diversity of the staphylococcal community occurring in both the environment and meat products of a small French unit, manufacturing traditional dry fermented sausages. The staphylococcal flora of the environment and meat products of this small unit was dominated by *S. equorum* and *S. succinus* species, rarely described in meat products and never in the environment. They represented the 49% and 33% of isolates, respectively. Other staphylococci belonged to the species *S. saprophyticus*, *S. xylosus*, *S. carnosus*, *S. simulans*, and *S. warneri.*

PCR-DGGE

In the last years, the single or combined use of sets of species-specific primers, started to be used with or be substituted by new, versatile approaches such as D/TGGE. The first paper, applying uniquely this technique for the identification of microorganisms involved in meat fermentations, was published by Cocolin, Manzano, et al. (2000). This work describes the use of PCR-TGGE to identify *Lactobacillus* spp. strains from naturally fermented Italian sausages. This method, based on the amplification of a small fragment from the 16S rRNA gene, followed by TGGE, allowed the differentiation of the strains of *Lb. sakei*, *Lb. curvatus*, *Lb. alimentarius*, *Lb. casei*, *Lb. plantarum*, and *Lb. brevis*.

The same authors (Cocolin, Manzano, Aggio, et al., 2001) used CNC control strains from international collections to optimize a PCR-DGGE method for their differentiation that was subsequently used to identify, based on co-migration with control strains, 90 isolates from natural fermented Italian sausages. The results showed that the strains of *S. xylosus*, *S. cohnii*, *S. intermedius*, *S. carnosus*, *K. varians*, and *K. kristinae* could be reliably identified on the basis of the DGGE mobility of the

16S rRNA gene V3 region; once again, *S. xylosus* was the main bacterium involved in fermented sausage production, representing, from the tenth day of ripening, the only CNC species isolated.

In the next years, PCR-DGGE, more than PCR-TGGE, found wide applications in studies of microbial ecology. These techniques showed the possibility to process high number of isolates in a shorter time; in fact, as already mentioned in the previous sections, these methods allowed grouping of strains and reduced the number of isolates to sequence.

Using these methods, Cocolin, Manzano, Cantoni, et al., (2001) monitored LAB dynamics in fermented sausages, at 3, 10, 20, 30, and 45 days of ripening, prepared in a local meat factory in Italy by traditional techniques. One-hundred and ninety two LAB strains were identified by PCR with the primers targeting the V1 region of the 16S rRNA gene and DGGE. Only two DGGE profiles were detected, belonging to *Lb. sakei* and *Lb. curvatus*. In the first stages of fermentation, *Lb. sakei* was the main LAB present, and only at the end of maturation *Lb. curvatus* became the predominant microorganism.

The same approach was applied to study the LAB and CNC ecology of fresh sausages stored at 4°C at days 0, 3, 6, and 10 (Cocolin et al., 2004; Rantsiou, Iacumin, et al., 2005). Strains with the same DGGE profile were grouped, and representatives of each group were amplified with primers targeting the V1–V3 region of the 16S rRNA gene and sequenced. A total of 80 LAB were analyzed, among which 69 isolates were identified as *Lb. sakei*. To study the *Staphylococcus* spp. ecology, PCR-DGGE was coupled with *S. xylosus*-specific primers because of the very similar DGGE migration patterns shown by strains belonging to *S. xylosus* and *S. haemolyticus*. Primers based on the *gehM* gene, coding for the lipase of *S. xylosus*, gave a definitive and sure identification of this species. Almost 50% of isolates were recognized as *S. xylosus*. Interesting population dynamics were observed, characterized by a succession of *S. pasteuri* and *S. warneri* at day 0, with *S. xylosus* at 3 days and *S. equorum* at 6 and 10 days.

Comi et al. (2005), studied three productions of a traditional fermented sausage of Northeast Italy. One-hundred and fifty LAB were isolated and identified by means of PCR-DGGE, as described above (Cocolin, Manzano, Cantoni, et al., 2001). The only species that were isolated in all three fermentations were *Lb. curvatus* and *Lb. sakei*. A low number of strains belonged to other species, namely *E. pseudoavium*, *L. lactis*, *Lb. brevis*, *Lc. mesenteroides*, *Lb. plantarum*, *Lb. paraplantarum/pentosus*, *Lc. citreum* and *W. paramesenteroides/hellenica*.

Rantsiou, Drosinos, Gialitaki, Urso, et al. (2005), using both species-specific PCR and PCR-DGGE, defined the geographic distribution of LAB populations responsible for the fermentation and maturation of naturally fermented sausages produced in three European countries: Greece, Hungary, and Italy. Three-hundred and fifty eight strains belonging to the LAB group were identified. Three species were common to all the three countries studied and were also the most numerous; *Lb. curvatus*, *Lb. plantarum*, and *Lb. sakei*. These three species were identified by species-specific PCR. Isolates that did not belong to any of three species above were identified by PCR-DGGE grouping and 16S rRNA gene sequencing.

Country specific-species: *E. faecium/durans*, *Lb. alimentarius*, *Lb. casei/paracasei,* were only found in sausages from Greece, *E. pseudoavium*, *L. lactis* subsp. *lactis* and *Lb. paraplantarum/pentosus* were only found in sausages from Italy, while *Lc. mesenteroides* and *W. viridescens* were specific to the sausages from Hungary. *Lb. paraplantarum* was isolated from both Italian and Greek products, and *Lb. paraplantarum/plantarum* was from both Greek and Hungarian sausages. *Lc. citreum* and *W. paramesenteroides/hellenica* were found in Italian and Hungarian products. A similar approach, coupling species-specific PCR with PCR-DGGE, was described by Urso et al. (2006), who investigated the ecology of the LAB microflora of three naturally fermented sausages produced in Northeast Italy. Four-hundred and sixty five strains were identified. The only species that were isolated in all three fermentations were *Lb. sakei* and *Lb. curvatus* (353 and 67 isolates, respectively). A low number of other LAB species were found as well, *Lb. plantarum*, *Lb. casei*, *Lb. paraplantarum*, *L. lactis*, *L. garvieae*, *Lc. mesenteroides*, *Lc. carnosum*, *Weissella hellenica*, and *W. paramesenteroides*.

Likewise, Iacumin, Comi, Cantoni, and Cocolin (2006b) used Six couples of species-specific primers (for *S. xylosus, S. epidermidis, S. simulan, S. carnosus, S. warneri*, and *K. varians*) to identify 617 CNC strains isolated from naturally fermented sausages in three different plants of the Northeast of Italy. Strains that did not give any PCR amplification with the species-specific primers were subjected to PCR-DGGE and sequencing of the V3 region of the 16S rRNA gene, as described by Rantsiou, Iacumin, et al. (2005). The same species of CNC were found in all the three processing plants, but their contribution to the fermentations was different. In two plants, *S. xylosus* was the main species involved in the fermentation process, while in the third the maturation was carried out equally by three species: *S. xylosus*, *S. warneri*, and *S. pasteuri*.

Cocolin, Urso, Rantsiou, Cantoni, & Comi (2006b) evaluated the ability of a commercial starter culture to perform a 28 day sausage fermentation, combining species-specific PCR and DGGE analysis. As was declared on the starter culture label, LAB isolated from the starter belonged to *Lb. plantarum* species. CNC strains of *S. xylosus*, together with *S. carnosus*, were also isolated and identified in disagreement with what was declared from the factory producing the starter.

Recently, PCR-DGGE was also used to investigate the yeast populations in Italian fermented sausages (Cocolin et al., 2006a). Yeast isolates were subjected to PCR-DGGE according to Cocolin, Bisson, & Mills (2000), and strains giving identical migration patterns were grouped together; at least two representatives were amplified with the primers NL1 and NL4 (Kurtzman, & Robnett, 1998) and sequenced. The work highlighted the dominance of *D. hansenii,* which was already present, as the major species, at the beginning of the fermentation. This species was usually accompanied by *Candida zeylanoides,* and only at the end of the fermentation, by *Metschnikowia pulcherrima*. At the start of the fermentation, a high biodiversity was observed, as five species could be identified; apart from *D. hansenii*, the species *Pichia triangularis*, *Candida parapsilosis*, *Saccharomyces cerevisiae*, and *Sterigmatomyces elviae* were found.

ISR-PCR

The polymorphism of the inter-gene spacer regions (ISR), was also used in combination with DGGE for the identification of the CNC strains isolated from fermented sausages. Blaiotta, Pennacchia, Ercolini, et al., (2003), applied a polyphasic molecular approach to describe staphylococcal population occurring in traditional fermented sausages of Southern Italy by combining DGGE and ISR-PCR. The results obtained by ISR-PCR and PCR-DGGE analysis allowed a clear diffentiation of the reference strains analyzed, with the exception of the pairs *S. cohnii-S. equorum* and *S. schleiferi-S. carnosus*. The strains that displayed the same ISR-PCR pattern could show a different PCR-DGGE profile and vice versa. Among the isolates, the most abundant species of CNC was *S. xylosus*. The strains of *S. saprophyticus*, *S. lentus*, *S. warneri*, *S. cohnii-S. equorum*, *S. epidermidis*, *S. haemolyticus*, *S. succinus*, *S. vitulus*, *S. pasteuri*, *S. aureus*, *M. caseolyticus*, and *Kocuria* spp. were also isolated.

In a previous research (Rossi et al., 2001), ISR-PCR permitted to successfully identify strains belonging to *S. xylosus*, *S. simulans*, and *S. carnosus* and other closely-related species, and *Kocuria* species, isolated from an Italian artisanal dry sausage. Fifty one CNC strains were genotypically identified by amplification with universal primers (Jensen, Webster, & Strauss, 1993), and once more, *S. xylosus* prevailed over the other CNC.

Recently, 240 CNC strains, isolated from the Spanish slightly fermented sausages, were subjected to ISR-PCR with universal primers (Martin, Garriga, et al., 2006). The results obtained by amplification of the intergenic regions were confirmed by species-specific PCR, as previously described (Aymerich et al., 2003). *S. xylosus* was the predominant species (80.8%), followed by *S. warneri* (8.3%), *S. epidermidis* (5.8%) *S. carnosus* (4.6%), and *K. varians* (0.4%).

Concerning ISR-PCR, discrepancy in the results is often found. While Forsman, Tilsala-Timisijarvi, & Alatossava, (1997), Mendoza, Meugnier, Bes, Etienne, & Freney, (1998), and Villard, Kodjo, Borges, Maurin, & Richard, (2000) reported a high polymorphism, Blaiotta, Pennacchia, Ercolini, et al., (2003), in agreement with Couto, Pereira, Miragaia, Sanches, & de Lancastre, (2001), found a lower discrimination potential of this analysis for staphylococci.

Molecular Methods for the Characterization of Isolated Strains

The growing interest in the studies of microbial ecology of fermented products and in monitoring of specific strains carrying out, spontaneously or after inoculation as starters, fermentation processes, pushed for the availability of methods for subspecies characterization of the isolates.

In 1998, RFLP analysis had been already used by Sanz et al. to characterize *Lb. sakei* strains isolated from dry-cured sausages, and previously identified by DNA-DNA hybridization. RFLP studies, by using the restriction enzymes *EcoRI*

and *HindIII*, and, as probes, cDNA from *Escherichia coli* 16S and 23S rRNA genes or from *Lb. sakei* 16S rRNA gene from *Lb. sakei* strains, showed distinct polymorphism levels. *EcoRI*-digested DNA, probed with the cDNA from *E. coli,* highlighted the presence of a unique cluster for the meat *Lb. sakei* isolates tested. When *HindIII*-digested DNA was hybridized with the *E. coli* cDNA probe, strain specific patterns were obtained, showing a higher discrimination power. Considerable strain differentiation was also observed when *EcoRI* and *HindIII* digests were hybridized with *Lb. sakei* 16S rRNA gene probes.

In the following years, RAPD-PCR became the most used technique to study the intra-specific variability even if, initially, it was applied for identification purposes (Andrighetto et al., 2001; Baruzzi, Matarante, Caputo, & Morea, 2006; Berthier & Ehrlich, 1999; Rebecchi et al., 1998). Rebecchi et al. (1998) studied the microbial community in the production of a 2-month-ripened Italian dry sausage. Their results, obtained by RAPD-PCR fingerprints, highlighted that environmental parameters interacted to select a limited number of strains during the fermentation process. The dominant strains belonged to the species *S. xylosus* and *S. sciuri*, among CNC, and *Lb. sakei* and *Lb. plantarum*, among LAB.

RAPD-PCR with primers M13 and D8635 was applied to identify 53 lactobacilli isolates originating from traditional fermented sausages and artisanal meat plants of Veneto region in Italy (Andrighetto et al., 2001). RAPD-PCR assigned most of the isolates to the species *Lb. sakei* and *Lb. curvatus* and, at the same time, an intra-specific variability was detected; in some cases, RAPD subgroups reflected the origin of the isolates; in others, different strains were found in the same environment.

In fermented products, the possibility to detect strains that were able to carry out the fermentation processes became extremely important in the selection of microbial strains to be used as starters. Rossi et al. (2001), compared and combined RAPD profiles, obtained with the primers OPL-01, OPL-02, OPL-05, and Hpy 1, from the *S. xylosus* strains isolated from dry sausages. In this paper, the authors underlined the suitability of the RAPD-PCR analysis to discriminate strains with technologically relevant activities; in fact, strains showing nitrate reduction and amino acid decarboxylase activities clustered separately.

Later, Baruzzi et al. (2006) correlated acidification, proteolytic, lipolytic, and nitrate reduction activities to strains isolated from "Salame di Senise",, a typical sausage produced in the South of Italy. The application of RAPD-PCR to more than 90 isolates made it possible to define 18 bacterial and 2 yeast bio-types, mainly belonging to three species of *Bacillus* (*Bacillus subtilis*, *B. pumilus* and *B. amyloliquefaciens*), three species of *Lactobacillus* (*Lb. curvatus*, *Lb. sakei* and *Lb. casei*), three species of *Staphylococcus* (*S. succinus*, *S saprophyticus*, and *S. equorum*), and *D. hansenii*.

The microbial community responsible for the artisanal fermentation of dry sausages produced in Argentina, was studied by Fontana et al. (2005). The authors carried out RAPD analysis with the primers M13, XD9, RAPD1, and RAPD2, to differentiate and characterize 100 strains of lactobacilli and CNC, identified by 16S rRNA gene sequencing. This approach allowed them to demonstrate that the ripening process of the Argentinean artisanal fermented sausages was driven by a limited number of *Lactobacillus* and *Staphylococcus* strains selected from the

environmental micro-biota for the ability to best compete under the prevailing conditions of the ecological niche. The authors concluded that these well adapted strains should have been eventually considered for the selection of starter cultures.

Cocolin et al. (2006b) used an identical approach to follow the development, in fermented sausages, of an inoculated commercial starter. RAPD characterization was carried out on 15 LAB and 15 CNC strains from a starter used in a sausage production and on 70 LAB and 70 CNC strains isolated during the same production. RAPD analysis with primer M13 revealed three *Lb. plantarum* RAPD bio-types, but only one of them was able to conduct the fermentation. *S. xylosus* strains from the starter culture, which clustered mainly with strains isolated at 14 and 28 days of ripening, were able to predominate only in the latter stages of fermentation. The different behavior of *Lb. plantarum* and *S. xylosus* could be explained considering the fact that the starter culture was dissolved in white wine, thus inhibiting the initial development of *S. xylosus*, not able to overcome the ethanol stress.

Recently, some authors evaluated possible correlations between microbial strains clustered on the basis of RAPD profiles and their manufacturing origin or geographical provenience. Comi et al. (2005), in the study of three fermentations of a Northeast Italy traditional fermented sausage, highlighted, by RAPD-PCR with primer M13, that lactobacilli population was distributed in a fermentation-specific way; the authors supposed that strains grouped in fermentation-specific clusters came from the ingredients used in the productions. *Lb. sakei* showed a higher degree of heterogeneity than *Lb. curvatus*.

The same approach was followed by Urso et al. (2006) who reached similar results. They isolated and identified, from three naturally fermented sausages produced in the Friuli-Venezia-Giulia region (Italy), 353 *Lb. sakei* and 67 *Lb. curvatus* strains. RAPD-PCR analysis by using primer M13 detected clusters formed by strains isolated from specific fermentations, confirming that ingredient composition, fermentation, and maturation parameters could play an important role in the selection of populations adapted to a specific environment. However, clusters containing strains isolated from different plants were also observed, underlining a homogeneous population distribution in the three different fermentations.

Rantsiou, Drosinos, Gialitaki, Metaxopoulos, et al., (2006) characterized, by RAPD-PCR with primer M13, *Lb. sakei*, *Lb. plantarum*, and *Lb. curvatus* strains isolated from naturally fermented sausages, produced in three different processing plants in continental Greece. In agreement with the results mentioned above, some strains were plant-specific, whereas others shared a degree of homology independently of the provenience.

In a similar study, *Lb. curvatus*, *Lb. plantarum*, and *Lb. sakei* strains, isolated from naturally fermented sausages produced in three European countries (Greece, Italy, and Hungary), were subjected to RAPD-PCR with primer M13 (Rantsiou, Drosinos, Gialitaki, Urso, et al., 2005). The distribution of the lactobacilli strains reflected, in almost all cases, the provenience of the same. It was thereby possible to distinguish the Italian, Greek, and Hungarian LAB populations.

In order to improve the characterization and differentiation among microbial strains, some authors considered both chromosomal and plasmidic characteristics.

Thus, 250 LAB isolated from low acid Spanish fermented sausages were analyzed (Aymerich et al., 2006) by combining RAPD-PCR and plasmid profiling. One-hundred and forty four different strains could be differentiated, 112 belonging to *Lb. sakei*, 23 to *Lb. curvatus*, and 9 to *Lc. mesenteroides*. The isolates identified as the same strain showed common phenotypic properties, in terms of biogenic amine production and antibiotic susceptibility, although isolates with specific traits could not be grouped in single clusters, indicating a probable horizontal exchange of DNA between bacteria.

Martin, Garriga, et al., (2006) also compared and combined RAPD-PCR and plasmid profiling techniques in the characterization of CNC strains isolated from Spanish slightly fermented sausages. In particular, the authors could differentiate 169 profiles out of the 194 *S. xylosus* isolates, indicating a great genetic intra-specific variability. It should be underlined that, in this study, the plasmid pattern analysis was more discriminatory than RAPD-PCR analysis; in fact, the first detected 140 profiles against the 118 of the latter. However, the number of different profiles obtained in the other CNC species analyzed at the strain level (*S. warneri*, *S. epidermidis*, and *S. carnosus*), was similar with both methods. Moreover, plasmid typing has two inconveniences: strains without plasmid cannot be typed, and many factors could influence the final plasmid patterns. For these reasons this analysis is considered not reliable when used alone.

In 2004, Cocolin et al. compared RAPD-PCR to rep-PCR technique, in order to characterize 69 *Lb. sakei* isolates, obtained from fresh sausages prepared by traditional techniques, and to detect different populations that eventually developed on different days of storage. Rep-PCR had been previously applied, in 2001, by Gevers, Huys, and Swings, to differentiate a wide range of lactobacilli recovered from the different types of fermented dry sausages, and it showed to be a promising tool for rapid and reliable speciation and typing of lactobacilli. In their research, Cocolin and his colleagues found that the *Lb. sakei* isolates were grouped in four RAPD clusters, whereas rep-PCR led to the identification of five different clusters. The authors underlined that most strains isolated at day 10 of ripening were grouped in a unique cluster, likely due to selection during storage at low temperature.

Once more RAPD-PCR and rep-PCR, together with Sau-PCR, were compared in a paper by Iacumin et al. (2006a). The purpose of this study was to characterize the *S. xylosus* strains isolated from naturally fermented sausages, produced in three different processing plants in the Friuli Venezia Giulia region (Italy), and eventually to detect a possible strain differentiation depending on their specific provenience. By applying the rep-PCR method, 10 clusters among a total of 13, were formed solely by strains coming from one plant, and some of these clusters contained a large number of isolates. Similar results were obtained with Sau-PCR, while the RAPD technique produced only 8 clusters, out of the 17, formed by strains coming from one plant. The authors confirmed the theory that, depending on temperature, humidity, and ingredients of specific production plants, there is always a specific selection of microorganisms which influences the characteristics of the final products. In this paper, the only correlation found between technological characterization and molecular typing was that all the strains isolated from one of the plants, grew at

a temperature of 10°C. The specific plant was the only one using low temperature maturation, thereby selecting a population of CNC that is able to grow under these environmental conditions.

Due to the efficiency and reliability of the technique, some authors used rep-PCR, exclusively. For example, Rantsiou, Iacumin, et al. (2005) used rep-PCR to characterize the *S. xylosus* strains isolated from fresh sausages, at 0, 3, 6, and 10 days. Molecular characterization and cluster analysis highlighted the presence of six main populations, but no correlation was obtained between the day of isolation and the grouping into clusters. This fact underlined a homogeneous distribution of the *S. xylosus* strains throughout the storage of the fresh sausages.

Lastly, RAPD-PCR analysis with primer M13 was used by Cocolin et al. (2006a) to characterize the *D. hansenii* strains, from Italian fermented sausages. The authors noticed a shift in the *D. hansenii* population from the beginning to the end of sausage maturation. In fact, strains present during the early stages of the fermentation were grouped in clusters that differed from those defined in the final phases of the maturation.

The last technique for molecular characterization that we take into account is PFGE. As reported in the previous sections, nowadays PFGE is considered the "gold standard" for the characterization of microbial strains, since it is very precise, reproducible, and reliable. However, few studies are reported where PFGE is applied to characterize LAB and CNC strains isolated from meat products, probably because it is a labor intensive and time-consuming technique. For this reason, PFGE is commonly applied in combination with other, PCR-based techniques, for strain typing. Di Maria et al. (2002) used RAPD-PCR analysis to monitor a *S. xylosus* starter culture throughout the ripening of "Soppressata molisana", a fermented sausage produced in Southern Italy. RAPD-PCR was successfully employed for the discrimination of the added strains from those naturally present during the ripening of the product. PFGE was then applied to confirm the RAPD results.

Pennacchia et al. (2006), coupled a species-specific multiplex PCR assay with PFGE analysis to identify and characterize 25 potential probiotic *Lactobacillus* strains isolated in a previous study, from fermented sausages.

In a study of the staphylococcal community occurring in the environment and in traditional dry fermented sausages produced in a small French unit, Corbiere Morot-Bizot, Leroy, & Talon, (2006) defined 17 distinct PFGE patterns named pulsotypes. Twelve of the pulsotypes (A to H, J, L, M, N) were found independently of the period of sampling (winter or spring), whereas the pulsotype P was sporadic in winter and the pulsotypes I, K, O, and Q were sporadic in spring. The 201 isolates of *S. equorum*, which dominated both in the environment and in the meat products, together with *S. succinus*, belonged to eight distinct unrelated pulsotypes (A to H), showing a wide diversity among this species. However, the dominant pulsotype C, with 77 clones, was found both in the environment of the small processing unit and in the meat products. The presence of a dominant pulsotype indicated that some strains could be capable for adaptation to food plant environment and processing. Recently, the same authors (Corbiere Morot-Bizot et al., 2007) monitored, by PFGE, the growth and survival

of *S. xylosus* and *S. carnosus* starters during sausage manufacture in two French processing plants. The PFGE analysis revealed that all *S. xylosus* and *S. carnosus* strains isolated corresponded to the starter strains inoculated. In particular, *S. xylosus* starter strain dominated the staphylococcal micro-biota, whereas the *S. carnosus* starter strain survived during the process. Since successful implantation of the starter cultures is obviously a pre-requisite for their contribution to sensorial qualities, this work highlighted both the efficiency and ineffectiveness of *S. xylosus* and *S. carnosus*, respectively, as starter cultures. It is noteworthy that neither of them were able to colonize the environment of the two processing plants.

The Microbial Ecology of Fermented Sausages as Determined by Culture-Independent Methods

In recent years, the study of the microbial ecology of fermented foods has been enhanced due to the introduction in this field of direct, culture-independent methods. These methods are based on the extraction of total nucleic acids from any given sample, and description of the microbial groups present in the foods (with identification of their individual members), based on DNA and/or RNA sequences.

The analysis of the nucleic acids can be carried out by hybridization with specific probes, by species-specific PCR or by universal PCR and sequence-based separation and identification of the PCR products. The disadvantage of the species-specific PCR is that there is a limit to the number of species one can detect/identify in a sample. Moreover, one has to know which microorganisms to look for in a sample. Alternatively, with the use of universal primers, theoretically all the species of large groups are amplified. Then, the sequence-based separation is achieved by D/TGGE. D/TGGE was first developed for the study of the microbial ecology in environmental samples (Muyzer & Smalla 1998), but soon found application in food microbiology (Ercolini, 2004).

The main advantages of the direct approaches are: (i) no cultivation takes place and therefore, the bias associated with the use of conventional microbiological media for enumeration/isolation, is negated, (ii) compared to the classic approach used so far in microbiology, that is based on isolation of strains from the food matrix and identification, either by physiological/phenotypical tests or by molecular methods, they are less time-consuming and require less effort, (iii) they allow a parallel description of the populations of different microbial groups. In contrast, these techniques generally require specialized personnel and relatively costly equipment. Furthermore, it has been determined that the detection limit for the most common method used in direct analyzes, that is the D/TGGE, is in order of 10^3–10^4 colony forming units (cfu)/g or ml (Cocolin, Manzano, Cantoni, et al., 2001). As a consequence, microbial groups that are present and active, but their population is lower than 10^3–10^4 cfu/g, will not be taken into consideration.

An interesting contribution to our knowledge of the microbial ecology of fermented products is provided by the application of the PCR-DGGE on the RNA extracted directly from the matrix and after reverse transcription. DNA may persist in any given environment, some times long after a microorganism is dead. In contrast, RNA is rapidly degraded after cell death and as a consequence, the application of RT-PCR-DGGE gives the fingerprint (or profiles) of alive and metabolically active populations. When RT-PCR-DGGE was applied in meat products, the results compared fairly well with those obtained by PCR-DGGE (Cocolin, Manzano, Cantoni, et al., 2001), although in certain cases the RT-PCR-DGGE profiles were richer (Cocolin et al., 2004).

These approaches have been successfully employed in the study of the microflora of fresh and fermented sausages. A scheme of the experimental approach that is followed is presented in Fig. 4.3, while in Table 4.2 the published studies employing

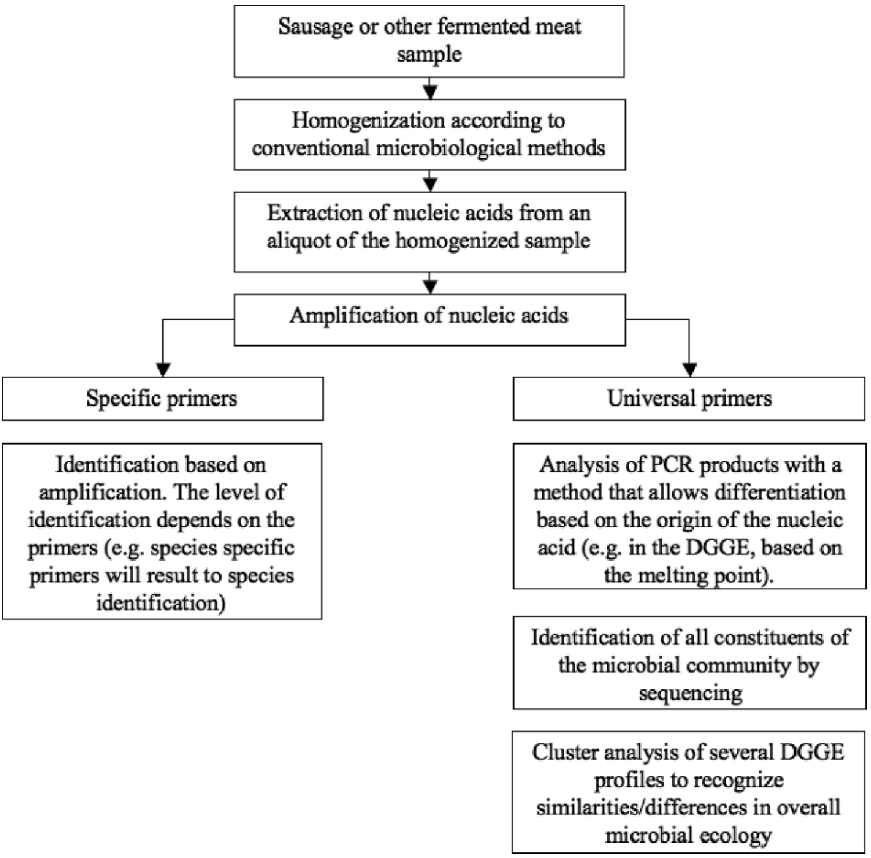

Fig. 4.3 Flowchart showing the experimental approach for the study of the microbial ecology in fermented sausages by using culture-independent methods.

Table 4.2 Meat products and information related to the studies performed with direct, culture-independent approaches

Type of product	Country	Direct Approach	Target group	Type of study	Reference
Fresh sausages	Italy	PCR-DGGE	LAB, CNC and yeasts	During storage	Cocolin et al., 2004
Fermented sausages	Friuli-Venezia-Giulia region, Italy	PCR-DGGE	LAB, CNC and yeasts	Throughout fermentation	Cocolin, Manzano, Cantoni, et al., 2001; Rantsiou et al. 2004; Rantsiou, Urso, et al., 2005
Fermented sausages (Ciauscolo salami)	Marche region, Italy	PCR-DGGE	LAB, CNC and yeasts	Final product	Silvestri et al., 2007
Fermented sausages (Sopressata)	Campania region, Italy	PCR-DGGE	LAB and CNC	Final product	Villani et al., 2007
Low- acid fermented sausages (fuets and chorizos)	Spain	Species-specific PCR	LAB and CNC	Final product	Aymerich et al., 2003
Artisanal dry sausages	Argentina	PCR-DGGE	LAB and CNC	Throughout fermentation	Fontana et al., 2005; Fontana et al. 2005
				Final product	Fontana et al. 2005

culture-independent methods to study the microbial ecology of these products are reported.

The Choice of PCR-DGGE Primers

When applying PCR-DGGE to study complex microbial populations, such as the ones present in food samples, an important parameter, that will influence the results obtained, is the choice of the amplification target gene prior to DGGE.

The target gene has to have two basic characteristics: (i) it should be present in all the members of the microbial group that is under consideration, (ii) it should have conserved regions, where universal primers can be designed, and variable regions, based on which separation is possible. In addition, if the RNA is going to be analyzed, the gene should be characterized by constitutive expression. Genes that fulfill these requirements are those that are involved in important and universal cell functions. Commonly, genes encoding for rRNA fall within this category. In bacteria, various regions of the 16S rRNA coding gene have been used in PCR-DGGE, while in yeasts, the 26S rRNA coding gene is commonly the target.

One important advantage of the 16S and 26S rRNA coding genes is the fact that, for both genes, databases of sequences from a large number of representative species is available. This is important since it allows identification of DGGE bands by sequencing and comparison with the database. A drawback associated with the use of rRNA coding genes is the inherent sequence heterogeneity, within the same species, that is the result of multi-copies of the genes with small differences in the sequence. The multi-copies often result in multi-bands in the DGGE profiles that complicate the analysis.

An alternative gene has been proposed for use in PCR-DGGE. It is the *rpoB* gene, encoding for the β-subunit of the RNA polymerase, which is usually present as a single copy in each genome. The limitation of using the *rpoB* gene is the restricted number of sequences available in the databases that hinders the identification of unknown DGGE bands.

In the DGGE studies performed so far on meat products, several primers have been used, and the relevant information is summarized in Table 4.3. The quality of information produced by PCR-DGGE is dependent on both the number and resolution of the amplicons in denaturing gradient gels (Yu & Morrison, 2004). In fingerprinting the microbial communities of a food product, it is important that the method allows differentiation of individual species that are associated with a specific food. For this reason, the primers used and the conditions employed in PCR-DGGE need to be carefully considered and if necessary, optimized, prior to application in real food samples (Cocolin, Manzano, Cantoni, et al., 2001; Yu & Morrison, 2004).

As shown in Table 4.2, for the profiling of bacterial populations in fresh and fermented sausages, several regions of the 16S rRNA coding genes have been employed.

Table 4.3 Summary of the target genes that have been employed in PCR-DGGE of meat products

Target group	Target region	Primer set	Primer Reference	Application reference
Bacteria	16S rRNA gene, V1	P1/P2	Klijn et al., 1991	Cocolin, Manzano, Cantoni, et al., 2001; Cocolin et al., 2004; Rantsiou, Urso, et al., 2005; Silvestri et al., 2007
	16S rRNA gene, V3	338f/518r	Ampe et al., 1999, Muyzer, de Waal, & Uitterlinden, 1993	Cocolin, Manzano, Cantoni, et al., 2001; Villani et al., 2007
	16S rRNA gene, V9	Ec1055/Ec1392	Ferris, Muyzer, & Ward, 1996	Cocolin, Manzano, Cantoni, et al., 2001
	16S rRNA gene, V2-V3	HDA1/HDA2	Walter et al., 2000	Cocolin, Manzano, Cantoni, et al., 2001
	16S rRNA gene, V6-V8	U968/L1401	Zoetendal, Akkermans, & de Vos, 1998	Cocolin, Manzano, Cantoni, et al., 2001
	16S rRNA gene, V3	P3/P4	Klijn et al., 1991	Cocolin, Manzano, Cantoni, et al., 2001
	16S rRNA gene, V3	V3f/Uni-0515r	Ercolini, Moschetti, Blaiotta, & Coppola, 2001b Lane, 1991	Fontana et al., 2005; Fontana et al., 2005
	16S rRNA gene, V2-V3	Bact-0124f/Univ-0515r	Lane, 1991	Fontana et al., 2005
	rpoB gene	*rpoB*1698f/*rpoB*2014r	Dahllof, Baillie, & Kjelleberg, 2000	Rantsiou et al., 2004
Yeasts/moulds	26S rRNA gene, D1-D2 loop	NL1-LS2	Cocolin, Bisson, et al., 2000	Cocolin et al., 2004; Rantsiou, Urso, et al., 2005; Cocolin et al., 2006a
	26S rRNA gene	U1-U2	Sandhu et al., 1995	Silvestri et al., 2007

In an early study, conducted by Cocolin, Manzano, Cantoni, et al., (2001) using control strains from culture collections that belonged to bacterial species commonly associated with meat products, it was concluded that region V1 of the 16S rRNA allowed the best differentiation in DGGE. In the DGGE optimization process with the P1/P2 set of primers, it was determined that the 40–60% denaturant gradient allowed differentiation of members of CNC, distinguishing *Kocuria* strains from *Staphylococcus* strains, while the 30–50% denaturant gradient allowed differentiation of all the *Lactobacillus* spp. tested. This set of primers has been subsequently used in DGGE profiling of bacterial populations in different meat products (Cocolin et al., 2004; Rantsiou, Urso, et al., 2005; Silvestri et al., 2007).

Fontana et al. (2005) applied a nested PCR approach in their study of fermented sausages. With primers P0/P4 (Klijn, Weerkamp, & de Vos, 1991), fragments of about 700 bp from the 5' region of the 16S rRNA coding gene were amplified and were used for nested PCR with two different sets of primers targeting the V2–V3 and the V3 regions. They concluded that the best DGGE patterns of the microbial community of fermented sausages were obtained with a 40–60% denaturant gradient for both primer combinations. In a subsequent study by Fontana et al. (2005), three sets of primers were tested, targeting the V1, V2–V3, and V3 regions, and the authors report that the best results from the direct analysis of the microbial community by DGGE were obtained by amplifying the V3 region.

Microbial Ecology of Meat Products Determined by Direct Approaches

Bacterial Ecology by PCR-DGGE

In the study of the microbial ecology of meat products, researchers have followed two approaches: they looked into the evolution of the different microbial populations throughout the production process and until the product was ready for marketing, or they considered the more static picture obtained from the final product. From the studies of the first type, a general observation is that a great diversity, both within the LAB and CNC groups, and complex DGGE profiles characterize the freshly prepared meat mix, just prior to the initiation of the fermentation or storage period, in the case of non-fermented meat products. This biodiversity and the associated complexity in the DGGE profiles, is sharply reduced from the first days of fermentation and just a few species appear to predominate and are commonly detected throughout the rest of the process. On the other hand, when a final product is taken into consideration, then the microorganisms that have managed to persist and most likely were responsible for the transformation process are detected. Of course, in this approach, microorganisms that may play a role at earlier stages but do not survive until the end, cannot be seen.

In the first work published (Cocolin, Manzano, Cantoni, et al., 2001) regarding direct DGGE analysis, traditional fermented sausages produced by local laboratories in the Friuli-Venezia-Giulia region of Italy were analyzed throughout fermentation

and ripening that lasted in total for 45 days. In the DNA and RNA-DGGE gels, multiple bands were visible for the first 3 days of fermentation, when different species, most of which were related to *Staphylococcus* spp. were identified. From the tenth day of ripening only the LAB bands were present. The LAB population was characterized by *Lb. sakei* and *Lb. curvatus* throughout the process. *Lb. plantarum* was only detected on the first day in the DNA gel and the band could have been generated from dead cells. *Staphylococcus* species were found only in the meat mixture before the sausages were filled and after 3 days. The only *Staphylococcus* species represented in the DGGE gel after 3 days was *S. xylosus*, which produced a specific band in the gel until the end of fermentation. The corresponding band in the RNA gel was only present at day 0 and day 3 and then disappeared. This could be explained by the large quantity of LAB RNA that restricted amplification of the *S. xylosus* RNA.

In a later study focusing on traditional fermented sausages from the same region of Italy, the goal was to follow the fermentation in three different plants, in which no starters are being used. PCR-DGGE was employed and the bands were identified by sequencing. Furthermore, cluster analysis of the DGGE profiles, obtained from the three fermentations and at different sampling points, was conducted (Rantsiou, Urso, et al., 2005). A general consideration that resulted from this study is that the main differences detected in the ecology, between the three sausages, were not represented by the species of microorganisms identified by band sequencing but by their relative distribution between the fermentations. In all the three fermentations, a stable signal, from the beginning of the period studied was visible for *Lb. curvatus* and *Lb. sakei*, which remained constant throughout the transformation. In one of the fermentation, a band that corresponded to *Lb. paracasei* was present for part of the period, while *L. garviae* was detected in two of the three sausages. No *Lb. plantarum* was detected in these sausages. As seen from the DGGE profiles, important contribution to the microbial ecology was given by the *Staphylococcus* species. *S. equorum* and *S. succinus* were present in all the fermentations, while *S. xylosus* was mainly present in one of them.

When the *rpoB* gene was targeted in PCR-DGGE of the same type of fermented sausages (Rantsiou, Comi, & Cocolin, 2004), significantly simpler profiles, all through the transformation process, were obtained. At the beginning of the fermentation, *S. xylosus* was detected while at the later stages, *Lb. curvatus*, *Lb. sakei*, and *Lb. plantarum* comprised the bacterial population.

In a study conducted by Fontana et al. (2005), PCR-DGGE has been applied to profile the bacterial community of the artisanal Argentinean sausages. Targeting the V3 region of the 16S rRNA coding gene, a highly complex fingerprint was obtained at day 0 of the fermentation that was characterized by the presence of *Lb. plantarum, P. acidilactici, Lb. sakei, Lb. curvatus, S. equorum* (identified by co-migration with control strains), and *Corynebacterium variabilis* (identified by sequencing). A more basic fingerprint was obtained at day 5 and day 14 (last day of ripening), with the presence of *Lb. plantarum*, *Lb. sakei*, and *Lb. curvatus,* while *S. saprophyticus* represented the *Staphylococcus* group. In a similar study by the same authors (Fontana et al., 2005), the fermentation of a sausage from the Tucumán region of Argentina

was followed during the 14 day production and the same sausage was compared to a ripened sausage from the Córdoba region. A high microbial diversity was observed at day 0 with the presence of *Lb. plantarum*, *Lb. sakei*, *S. saprophyticus*, *C. variabilis* and that with varying intensities were also present in the other two sampling points (day 5 and day 14). When the two sausages were compared, a common band, associated with *Lb. sakei* was detected. Furthermore, the bacterial community of the Tucumán sausage contained *S. saprophyticus* and an uncultured bacterium while in the Córdoba sausage *Brochothrix thermosphacta* was present.

The bacterial ecology of the traditional Italian salami "ciauscolo" was recently surveyed by PCR-DGGE (Silvestri et al., 2007). The V1 region was amplified from 22 samples of 14 artisan and 8 industrial production plants from different geographical areas of the Marche region, in Central Italy. Overall, five LAB species were detected: *Lb. plantarum*, *Lb. curvatus*, *Lb. sakei*, *P. acidilactici*, and *L. lactis*. No *Staphylococcus* bands were observed in the DGGE profiles, either because the populations were below the detection limit or due to the higher number of LAB that may create a masking effect. The most frequently detected species were, once again, *Lb. curvatus* and *Lb. sakei*.

Finally, in a recent study of the ecology of a fermented sausage from Southern Italy, the "soppressata", targeting the V3 and V1 regions, it was possible to identify *S. xylosus*, *S. succinus*, and *S. equorum* among the staphylococci and *Lb. sakei* and *Lb. curvatus* within the lactobacilli (Villani et al., 2007).

A slightly different picture of bacterial ecology was seen when fresh sausages from Italy were considered (Cocolin et al., 2004). These products are highly perishable, since no fermentation takes place. After mixing of the ingredients and casing, they are stored at 4°C. They have a very short shelf life (about 10 days) and are cooked before consumption. Bacterial DGGE profiles were characterized, for both DNA and RNA, by the presence of a constant band throughout the process that was *B. thermosphacta*, whereas the *Lactobacillus* spp., visible at the DNA level, were only marginally present at the RNA level, mainly represented by *Lb. sakei*. *Lc. mesenteroides* was present at both DNA and RNA the last day of sampling. On the fresh mix, at the RNA level, a *Bacillus* sp. band was present, while *Staphylococcus* spp. was also detected during storage of the fresh sausages.

An alternative application of the PCR-DGGE directly in a food matrix was presented by Cocolin et al. (2006b). The authors followed the microbial dynamics of sausage fermentation, with particular interest for *Lb. plantarum* and *S. carnosus* that were inoculated as a starter. PCR-DGGE allowed the detection of a stable band, identified by sequencing as *Lb. curvatus*, that was only marginally detected by the conventional microbiological techniques. This application shows the potential of the method in monitoring, in a culture-independent way, specific microbial groups.

Yeast Ecology by PCR-DGGE

The yeast ecology has been described by targeting the 26S rRNA coding gene for Italian fermented sausages of the Friuli-Venezia-Giulia region (Rantsiou, Urso,

et al., 2005; Cocolin et al., 2006a) and of the Marche region (Silvestri et al., 2007) and for fresh sausages (Cocolin et al., 2004).

Overall, the DGGE profiles are less complex than those obtained for the bacteria and oftentimes, they are characteristic of the products. In particular, when a cluster analysis of the yeast DGGE profiles was carried out, from the samples collected during production of three fermented sausages, a fermentation-specific distribution was obtained, with the clusters containing samples from the same fermentation (Rantsiou, Urso, et al., 2005). In this study, the yeast ecology results were characterized by members of the genera *Candida*, *Debaryomyces*, and *Willopsis*. *D. hansenii*, a proteolytic yeast that is associated with fermented sausage production, was dominant in one of the three fermentations but was not seen in the other two productions. *C. krisii* and *Willopsis saturnus* and *C. sake* or *austromarina* characterized, respectively, the other two fermentations. Finally, one band was common in all the three fermentations that after sequencing showed a 86.5% homology to *Mayaca fluviatilis*. This homology score is very low and most likely, the DGGE band corresponds to a yeast species that has not yet been described or for which no 26S rRNA gene sequence is available.

The predominance of *D. hansenii* was also confirmed by a later study in Italian sausages fermented at low temperatures, probably due to its physiological characteristics, and it should therefore be considered well adapted to the specific environment yeast species (Flores, Durà, Marco, & Toldrà, 2004). Throughout the 60 days of fermentation a stable band, at both the DNA and RNA level, identified as *D. hansenii*, was visible in the DGGE gels (Cocolin et al., 2006a).

In the "ciauscolo" salami of the Marche region, the most frequently detected species was *D. hansenii*, while *C. physchrophila* and *Saccharomyces barnettii* were also occasionally found (Silvestri et al., 2007).

D. hansenii was the yeast, characterizing the DNA and RNA profiles of the fresh sausages. It was present during the whole period followed. The only other yeast species detected was *Capronia mansonii*, at day 0 and only in the RNA profile (Cocolin et al., 2004).

Bacterial Ecology by Species-Specific PCR and qPCR

Aymerich et al. (2003) studied the bacterial ecology of 17 commercially available low acid fermented sausages from Spain. Ten of them were fuets (cold-ripened fermented sausages with black pepper) and seven where chorizos (cold-ripened fermented sausages with red pepper). They used 12 sets of primers for 12 different species, six LAB (*Lb. sakei, Lb. curvatus, E. faecium, Lb. plantarum, L. lactis*, and *P. acidilactici*) and six CNC (*S. carnosus, S. warneri, K. varians, S. xylosus, S. simulans*, and *S. epidermidis*). They screened for the 12 species in the fermented sausage homogenate, before and after a 24-hour enrichment period (in MRS for the LAB and in mannitol salt broth [MSB] for the CNC).

Without enrichment, *Lb. sakei* and *Lb. curvatus* were detected in 11.8% of the samples, and *Lb. plantarum* and *S. xylosus* were detected in 17.6%. The low percentages of detection for the species of importance in the fermentation is probably

related with a low sensitivity limit of the method, that needed an enrichment step, in order to obtain some information for the species present. However, it should be noted that enrichment can significantly alter the picture of the microbial ecology, since different microbial groups or even species within a microbial group, have different abilities to grow in enrichment media. After the 24h enrichment, it was possible to detect *Lb. sakei* and *S. xylosus* in all the samples, a result that highlights that these two species are predominant in slightly fermented sausages. Regarding other LAB species, *Lb. curvatus* was detected in 71% of the samples (in 80% of the fuets and 57% of the chorizos), while *Lb. plantarum* was present in all of the chorizos and in 50% of the fuets (62% of the samples in total). *P. acidilactici* and *L. lactis* were not detected while *E. faecium* was present in 11.8% of the samples. With regard to the other CNC species, *S. carnosus* was only detected in chorizos (in 14% of them) and *S. epidermidis* in the fuets (in 20% of them). No *S. simulans* or *S. warneri* were found. Overall, the ecology of the low-acid fermented sausages from Spain, as determined after the enrichment, was characterized by the presence of *Lb. sakei* and *S. xylosus*. *Lb. curvatus* and *Lb. plantarum* were also detected but with different frequencies for the two types of sausages. In addition, the presence of *S. epidermidis* and *S. carnosus* characterized the fuets and the chorizos respectively, although they were not detected in all the respective samples.

Recently, a protocol based on qPCR amplification of the 16S-23S rRNA intergenic transcribed region (ITS) of *Lb. sakei*, has been optimized (Martin et al., 2006). The goal of the work was to develop a tool that would allow the quantification of *Lb. sakei*, without cultivation, in the fermented sausages. The protocol was tested in 11 commercial samples of sausages and meat batter; and *Lb. sakei* was detected in the range of 3 to 9 $\log_{10}$ cfu/g. Of the 11 samples, *Lb. sakei* was not detected in three while in a fourth, it was below the quantification limit, which was determined to be in the order of 10^5 cfu/g. The method can be considered as a valid alternative to conventional microbiological testing of fermented sausages that allows rapid and accurate quantification of *Lb. sakei*.

Conclusions

Often at times, when both the culture-dependent and independent methods are applied in parallel to study the microbial ecology of fermented foods, interesting information becomes available. From the experience gained so far in this field, we can conclude that the two approaches complement each other, and their concurrent use highlights important aspects that could otherwise pass un-noticed.

Indisputably, the main breakthrough of the direct methods is that, the inherent limitations of microbial cultivation, related to non-culturable organisms, either due to injury or stress, or due to the selectivity of the media, are circumvented. Furthermore, for the first time, we are able to see in situ which microorganisms are metabolically active and derive information regarding the relative importance of different microbial groups in the transformation process.

The results of the application of molecular approaches to study the microflora of fermented sausages that have been presented here, highlight the unequivocal dominance of *Lb. sakei* and *Lb. curvatus* in these types of products. To a lesser extend, *Lb. plantarum*, in a more product-specific manner, was also shown to be important. The presence and activity of the different *Staphylococcus* members appears to be product-specific as well. Finally, regarding yeasts, *D. hansenii* was detected with a high frequency (Rantsiou & Cocolin, 2006).

Moreover, the use of molecular techniques, able to differentiate strains within the same species, helps in the understanding of the intra-species diversity that may characterize fermented products produced in different countries or in different plants within the same country. Different strains, together with the different ingredients used and the technological processes during production, are responsible for the development of specific sensory profiles. To us, this is an important aspect to be considered in our understanding of the fermentation process, and it needs to be investigated further. Defining and understanding microbial dynamics, as determined by species successions, as well as microbial ecology, as determined by species interactions at each time point and throughout fermentation, is crucial, since these are the parameters that will have a great impact on the organoleptic and sensorial characteristics of the final product.

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