Available online at www.sciencedirect.com
SCIENCE DIRECT®
International Journal of Food Microbiology 108 (2006) 255 – 267
INTERNATIONAL JOURNAL OF Food Microbiology
www.elsevier.com/locate/ijfoodmicro

New developments in the study of the microbiota of naturally fermented sausages as determined by molecular methods: A review

Kalliopi Rantsiou, Luca Cocolin *

Dipartimento di Scienze degli Alimenti, Facolta' di Agraria, Universita' di Udine, via Marangoni 97, 33100, Udine, Italy

Received 28 December 2004; received in revised form 5 June 2005; accepted 17 November 2005

Abstract

The microflora of different types of fermented sausages has been defined by isolation and biochemical identification of the microorganisms commonly found in these products. It is generally agreed that the main microbial groups involved in such products are lactic acid bacteria and coagulase-negative cocci. In addition, and depending on the product, other groups may play a role, such as yeasts and enterococci. Since it appears that the types of microbial groups, or even the specific strains of a given microbial group, that dominate the fermentation, significantly affect the organoleptic profile of the final product, there is an increasing interest in the description of the microbiota that are found in different fermented sausages. More recently, new tools, based on molecular methods, allowing fast and unequivocal identification of strains, isolated from fermented sausages, became available. These methods have been successfully applied and, in general, biochemical and molecular identification compared well. However, new information comes to light when molecular methods are applied to DNA and/or RNA extracted directly from sausages. This approach eliminates problems related to traditional isolation. This review deals with the recent findings and results of the application of molecular methods, in a culture-dependent and culture-independent manner, on the study of the microflora of fermented sausages.
© 2006 Elsevier B.V. All rights reserved.

Keywords: Fermented sausages; Molecular methods; Ecology; PCR; RAPD; DGGE

1. Introduction

Meat fermentation is an ancient process originally used to extend the shelf life of perishable raw materials. During fermentation, complex biochemical and physical reactions take place that result in a significant change of the initial characteristics. Moreover, production of aromatic substances during fermentation define the sensorial characteristics of the final product that are significantly different from the ones of the raw materials used. Low acid fermented meat products (final pH 5.3 to 6.2) (Aymerich et al., 2003) are a group of traditional Mediterranean products with a great diversity between different countries and between different regions of the same country. The first evidence of sausages production date back to the period of the Roman empire (Lücke, 1974).

Fermentation of sausages is a well-known microbial process, and pioneering contributions to the study of the ecology during ripening are available from the 1960s (Lerche and Reuter, 1960; Reuter, 1972; Lücke, 1974). These studies stated that lactic acid bacteria (LAB, *Lactobacillus* spp.) and coagulase-negative cocci (CNC, *Staphylococcus* and *Kocuria* spp.) are the two main groups of bacteria that are considered technologically important in the fermentation and ripening of sausages.

LAB are usually present in high hygienic quality raw meat at low numbers (10^2–10^3 colony forming units, cfu/g), but they rapidly dominate the fermentation due to the anaerobic environment and the presence of NaCl, nitrate and nitrite, conditions that favour their growth. They reduce the pH of the sausage with their main metabolic activity, the production of lactic acid from carbohydrates (Hammes et al., 1990; Hammes and Knauf, 1994). In addition to lactic acid production, LAB are responsible for the “tangy” flavour of sausages, and for the small amounts of acetic acid, ethanol, acetoin, pyruvic acid and carbon dioxide (Demeyer, 1982; Bacus, 1986). Recently, it was demonstrated that *Lactobacillus* spp. commonly isolated from fermented sausages possess proteolytic activity on muscle sarcoplasmatic proteins (Fadda et al., 1998, 1999a,b, 2002; Sanz et al., 1999; Pereira et al., 2001).

* Corresponding author. Tel.: +39 0432 590 759, +39 0432 590 730; fax: +39 0432 590 719.
E-mail address: lscocolin@uniud.it (L. Cocolin).

0168-1605/$ - see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijfoodmicro.2005.11.013

CNC participate in the development and stability of a generally appreciated red colour through nitrate reductase activity that eventually leads to the formation of nitrosomyoglobin. Furthermore, nitrate reduction produces nitrite that can limit lipid oxidation (Talon et al., 1999). By the activity of CNC, different aromatic substances and organic acids are also produced. In particular, proteolysis and lipolysis influence both texture and flavour development due to the release of low molecular weight compounds, including peptides, amino acids, aldehydes, amines and free fatty acids, which are important flavour compounds, or precursors of flavour compounds (Demeyer et al., 1986; Schleifer, 1986).

The type of microflora that develops in sausage fermentation is often closely related to the ripening technique utilised. Sausage with a short ripening time have more lactobacilli from the early stages of fermentation, and an "acid" flavour predominates in the products, which are commonly sold after less than two weeks of ripening. The intensity of this flavour depends on the pH value, but, at a given pH, a high amount of acetic acid gives the product a less "pure" and more "sour" flavour (Montel et al., 1998). Longer ripening times and greater activity of microorganisms other than LAB, such as CNC and yeasts, lead to higher levels of volatile compounds with low sensory thresholds. Lipids and peptides are precursors of most of these substances. Tissue enzymes are the main agents of initial lipolysis and proteolysis processes (Lücke, 2000), however, later in ageing, bacterial enzymes play a role in the degradation of peptides formed (Molly et al., 1997).

In some fermented sausages, particularly those produced in France, Spain and Italy, the sensory properties of the products are also influenced by the development of the surface flora, consisting of moulds and yeasts (Lücke, 2000).

In naturally fermented sausages there is an evident and strong connection between the microflora that develops during transformation and the sensory characteristics of the final product. As a consequence the study of the microbial ecology is an important parameter to consider in sausage fermentation and for this reason is a subject of intense study.

The information collected so far on the ecology of fermented sausages was obtained by using traditional microbiological methods, based on plating analysis and biochemical identification of isolated strains. With these techniques only easily culturable organisms are isolated, and often microorganisms for which selective enrichment and subculturing is problematic or impossible cannot be characterised. The introduction in the field of food microbiology and food fermentation of new approaches, exploiting molecular methods, complements the studies carried out so far and allows scientists to overcome the limitations of traditional methods.

The goal of this paper is to report the latest findings in the study of the microbial ecology of fermented sausages by using molecular methods, with a special focus on LAB and CNC microbial groups. A comparison of the microbial ecology picture, as determined by traditional methods and molecular methods, will also be presented and discussed.

2. Experimental approaches to study the microbial ecology in fermented sausages

The main differentiation of the experimental approaches that scientists used so far for the study of the ecology of fermented sausages is between culture-dependent and independent methods. Moreover, the methods exploited to achieve the result of understanding the microbial populations involved in the transformation process can be distinguished in traditional and molecular ones. A schematic representation of the interconnection between these approaches is presented in Fig. 1.

2.1. Culture-dependent methods

The culture-dependent methods are based on growth of the bacterial species on synthetic media that resemble the conditions of the system from which the microorganisms are isolated. In the case of fermented sausages, it has been defined that the de Man, Rogosa, Sharpe (MRS, de Man et al., 1960) medium is the most suitable for the isolation of LAB, while for the CNC the Mannitol Salt Agar (MSA) (APHA, 1966) is often used for its characteristics of selectivity towards halotollerant species. The microbiological technique used for the sampling is carried out with the goal to facilitate the growth of the desired species. For instance, for the LAB the double layer technique is used, where the appropriate dilution of the sample is incorporated in the medium kept at 45–50 °C, and after solidification of the first layer a second is added, while for the CNC, a simple spread on the surface of the plate is sufficient. In the first case, a microaerophilic environment is created that makes LAB growth optimal, in the second case, the aerobic nature of CNC is exploited to enhance their growth. The following steps of the culture-dependent technique concern the isolation, after counting, and the biochemical identification. A first problem that scientists have to face is the selection of the colonies to isolate. Sometimes it is possible to observe differences in the morphology and colour of the colonies (i.e. on MSA, *Kocuria* spp. have a pale red colour, while *Staphylococcus* spp. appear as yellowish, due to their capability to produce acids from mannitol, thereby changing the pH and the colour of the phenol red contained in the medium), but almost all of the times they appear as identical. For this reason, the approach more often used is the random isolation. Finally, the study of the biochemical characteristics, such as the capability to produce acids from different carbohydrates, is performed to achieve the identification of the isolated microorganisms. These tests can be prepared "in house", or purchased from commercial facilities (Biomerieux, API Tests). A big hurdle to overcome, when applying biochemical tests, is their interpretation. In both cases of "in house" and commercial, the positivity to a specific character is given by a change in the original colour of the medium. For this reason, the subjective interpretation by the technician becomes an important factor, and often adequate identification (e.g. to the species level) is not achieved using this approach (Samelis et al., 1994; Coppola et al., 2000). Moreover, biochemical identifications are time-consuming.

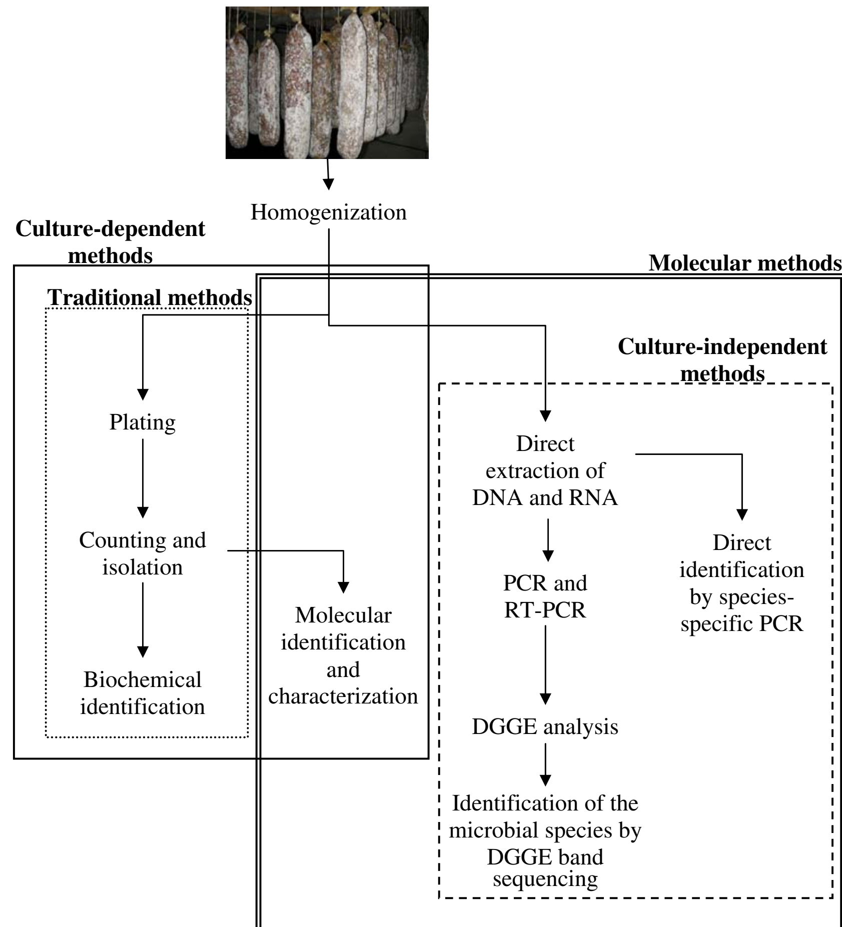

Fig. 1. Schematic representation of the experimental approaches used for the analysis of the microflora in fermented sausages.

Alternative to traditional methods for identification of isolated strains, molecular methods have attracted the attention of many researchers in the last 10 years. They are reproducible, easily automated and rapid (Charteris et al., 1997). The increasing availability of the sequences of the 16S rRNA gene (Collins et al., 1991) and the intergenic region between 16S rRNA and 23S rRNA genes (Nour, 1998) allowed the development of different methods for the identification of microbial species of interest in the field of sausage fermentation. Ribosomal RNA probes (Nissen and Dainty, 1995; Hertel et al., 1991), species specific PCR primers (Berthier and Ehrlich, 1998; Yost and Nattress, 2000; Rossi et al., 2001; Blaiotta et al., 2003b; Morot-Bizot et al., 2003), randomly amplified polymorphic DNA (RAPD)-PCR analysis (Berthier and Ehrlich, 1999; Andrigetto et al., 2001), restriction fragment length polymorphism (RFLP) analysis of the 16S rRNA gene (Sanz et al., 1998; Lee et al., 2004), multiplex PCR (Corbiere Morot-Bizot et al., 2004), ribotyping (Zhong et al., 1998), PCR amplification of repetitive bacterial DNA elements (rep-PCR) (Gevers et al., 2001), temperature gradient gel electrophoresis (TGGE) (Cocolin et al., 2000) and denaturing gradient gel electrophoresis (DGGE) (Cocolin et al., 2001a; Ercolini et al., 2001a; Blaiotta et al., 2003a) have been applied for the identification of LAB and CNC isolated from fermentation of sausages. Moreover, other molecular techniques were described as valuable tools for the same purposes. Samelis et al. (1995) developed a sodium dodecyl sulfate-polyacrylamide gel electrophoresis of whole-cell proteins to differentiate between *Lactobacillus sakei* and *Lactobacillus curvatus*, isolated from naturally fermented Greek dry salami and Di Maria et al. (2002) used pulsed field gel electrophoresis (PFGE) to monitor *Staphylococcus xylosus* DSM 20266 added as a starter during fermentation and ripening of soppressata molisana, a typical Italian sausage.

2.2. Culture-independent methods

As mentioned above, conventional microbiological methods for the characterisation of microorganisms that require selective enrichment and subculturing are problematic or impossible. In the last decade it was shown that classical microbiological techniques do not accurately detect microbial diversity (Hugenholtz et al., 1998; ben Omar and Ampe, 2000) and as a consequence, an increasing interest in the development and use of culture-independent techniques was shown. A variety of new methods has been developed to directly characterise the microorganisms in particular habitats without the need for enrichment or isolation (Head et al., 1998). Typically, these strategies examine the total microbial DNA (or RNA) derived from mixed microbial populations to identify individual constituents (Hugenholtz and Pace, 1996).

This approach eliminates the necessity for strain isolation, thereby negating the potential biases inherent to microbial enrichment. Studies, which employed such direct analysis, have repeatedly demonstrated a tremendous variance between cultivated and naturally occurring species, thereby dramatically altering our understanding of the true microbial diversity present in various habitats (Cocolin et al., 2002a; Cocolin and Mills, 2003).

One culture-independent method for studying the diversity of microbial communities is analysis of PCR products, generated with primers homologous to relatively conserved regions in the genome, by using DGGE or TGGE (Muyzer et al., 1995; Heuer and Smalla, 1997). These approaches allow separation of DNA molecules that differ by a single base (Myers et al., 1987) and hence have the potential to provide information about variations in target genes in a bacterial population. By adjusting the primers used for amplification, both major and minor constituents of microbial communities can be characterised. Different authors (Muyzer and Smalla, 1998; Muyzer, 1999; Ercolini, 2004) have reviewed the application of DGGE in the field of microbial ecology. In general, the technique is based on the electrophoretic separation of PCR-generated double stranded DNA in a polyacrilamide gel containing a gradient of chemical denaturants (urea and formamide). As the DNA molecule encounters an appropriate denaturant concentration, 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. For a mix of DNA molecules of different sequences, a band pattern is obtained that is representative of the sampled microbial community. In the TGGE method, the temperature is the main denaturing agent.

Modern image analysis systems have proven to be of value for the analysis of DGGE bands and their associated patterns. For instance, pairwise matching of DGGE bands in separate gel lanes has facilitated the calculation of similarity coefficients to describe relationships between communities (van der Gucht et al., 2001).

In the early 90s, Muyzer et al. developed DGGE with the potential to characterise the microbial flora quickly and economically. It was developed for the study of microbial ecology in environmental ecosystems, such as communities of sulphate-reducing bacteria (Muyzer et al., 1993). Only later, the same approach started to be considered in the field of food fermentations and this resulted in an impressive number of papers using DGGE published in the last 4 years. Cheese (Ercolini et al., 2001b, 2003, 2004; Randazzo et al., 2002), sausage (Cocolin et al., 2001c, 2004; Rantsiou et al., 2005c), wine (Cocolin et al., 2001b; Mills et al., 2002), sourdough (Meroth et al., 2003a,b) and malt whisky (van Beek and Priest, 2002) fermentation processes have been studied using this approach. DGGE was also applied to study the ecology of *Listeria* spp. (Cocolin et al., 2002b) and *Yersinia* spp. (Cocolin and Comi, 2005) without traditional isolation and identification.

A second culture-independent method used to study microbial ecology is represented by direct PCR and reverse transcription (RT)-PCR amplification using species-specific primers. The possibility to exploit the potential of polymerase chain reaction to amplify, theoretically, a single nucleic acid molecule allows the detection of low-number populations that may be lost when traditional methods, such as plating or selective enrichments, are used. Species-specific PCR is a rapid and reliable molecular technique for the characterisation of bacterial communities without colony isolation. However, the sensitivity of PCR in foods can be reduced due to the complexity of the food matrix and the presence of many PCR inhibitors. Many substances have been proven to be PCR inhibitory (Buffone et al., 1991; Rossen et al., 1992; Akane et al., 1994; Powell et al., 1994) and for this reason appropriate DNA and RNA extraction protocols, chosen on the basis of the food matrix under study, are used to avoid non-amplification of microbial species actually present in the ecosystem investigated.

Lastly, fluorescence in situ hybridisation (FISH) represents a new culture-independent technique in the field of food fermentations. Despite the considerable background of knowledge, its application to the study of the distribution of microbial populations in food has been limited (Ercolini et al., 2003).

3. Study of the fermented sausages microflora by traditional culture-dependent methods

The study of the fermented sausages microflora by traditional methods has been carried out by different authors in the last 20 years. In some cases, only the technologically relevant microbial groups were examined (LAB and CNC), whereas other studies reported the dynamics of several microbial populations, thereby carefully profiling the changes in the microbial ecology during fermentation and ripening. LAB and total aerobic microbes are the fastest growing during the production of sausages. From the initial counts of 10^2–10^3 cfu/g, they reach values of 10^7–10^8 cfu/g in the first three days of fermentation (Sanz et al., 1988; Samelis et al., 1993, 1998; Rebecchi et al., 1998; Cocolin et al., 2001a,c; Metaxopoulos et al., 2001; Papamanoli et al., 2002, 2003; Aymerich et al., 2003; Mauriello et al., 2004; Drosinos et al., 2005), and this situation establishes both in the core and in the external layers of the sausage (Coppola et al., 2000). Their counts remain quite stable in number throughout the ripening period. Also the CNC show a rapid increase in the first days of fermentation, but in some cases it has been reported that fast growth of LAB, with the consequence of deep acidification of the substrate, could result in inhibitions towards CNC, that exhibit a slow growth (Papamanoli et al., 2002, 2003). However, in several studies it was shown that CNC, together with LAB are the populations numerically more important at the end of the ripening period (Rebecchi et al., 1998; Cocolin et al., 2001a; Aymerich et al., 2003; Mauriello et al., 2004). Yeasts only in few cases were found to be relevant to sausage fermentation. Samelis et al. (1993) determined a stable number of 10^5 cfu/g in all stages of production, and this data was confirmed by Metaxopoulos et al. (2001). Coppola et al. (2000) found yeasts to be a predominant flora, together with LAB and CNC, in Naples-type salami, with *Debaryomyces* spp., described as a proteolytic agent during

fermentation of sausages (Santos et al., 2001), as the main representative. Significant differences on the presence and persistency of enterococci were reported by different authors. In some studies, after an increase to 10^5 cfu/g up to 20 days of fermentation, a reduction in the numbers was observed, leading to a final count of about 10^2 cfu/g (Cocolin et al., 2001a,c; Aymerich et al., 2003; Papamanoli et al., 2003). On the other hand, several papers reported a stable population of enterococci of about 10^5–10^6 cfu/g at the end of the fermentation (Rebecchi et al., 1998; Samelis et al., 1998; Metaxopoulos et al., 2001; Comi et al., 2005; Rantsiou et al., 2005c), becoming an important population possibly influencing the final organoleptic characteristics of the product. Since enterococci are able to produce ammonia and other amines, they possibly contribute to the final flavour of the product.

Total enterobacteria and *Escherichia coli* are usually counted only in the first days of fermentation and their number decreases due to the acidification performed by LAB (Rebecchi et al., 1998; Cocolin et al., 2001a,c; Metaxopoulos et al., 2001; Aymerich et al., 2003; Papamanoli et al., 2003; Drosinos et al., 2005). Fermented sausages are usually free of sulphite-reducing clostridia and coagulase-positive staphylococci (Samelis et al., 1998; Cocolin et al., 2001a,c; Papamanoli et al., 2003; Aymerich et al., 2003; Comi et al., 2005; Drosinos et al., 2005; Rantsiou et al., 2005c), but in some studies suspected colonies of *Staphylococcus aureus* were isolated from the meat mix (Rebecchi et al., 1998; Metaxopoulos et al., 2001).

Considering the safety aspect of the fermented sausages, several studies investigated the presence of *Listeria monocytogenes* and *Salmonella* spp. during the different stages of the production. It has been defined that generally these foodborne pathogens are absent in 25 g of product (Aymerich et al., 2003; Comi et al., 2005; Rantsiou et al., 2005c). *Listeria monocytogenes* can contaminate the fresh sausage mix, but commonly it is undetectable at the end of the fermentation (Samelis et al., 1998; Metaxopoulos et al., 2001; Drosinos et al., 2005).

4. Identification of LAB and CNC populations from fermented sausages

Different studies focusing on the identification of LAB and CNC during sausage fermentation are available. The strains, isolated at different steps of the transformation process, were identified by biochemical tests, as reported above, but, more recently, molecular methods were used for the same purpose.

4.1. Identification by physiological and biochemical tests

The studies that applied biochemical tests to achieve the identification of LAB and CNC and the results obtained are reported in Table 1. As shown, the strains were mainly isolated from fermented sausages produced in Greece, Italy and Spain, but also a study on the identification of CNC from French sausages is available (Montel et al., 1993). Only in a few cases, both LAB and CNC were taken into consideration (Samelis et al., 1998; Coppola et al., 2000), while more often researchers

Table 1
LAB and CNC isolated from naturally fermented sausages from different countries and identified by traditional methods

Country	Traditional product	Bacterial species	Reference
Greece	Dry salami	*L. curvatus* *L. sakei* *L. plantarum* *L. farciminis* *L. coryniformis* *L. casei* subsp. *pseudoplantarum* *W. viridescens* *W. hellenica* *W. paramesenteroides*	Samelis et al., 1994
	Dry salami	*L. sakei* *S. saprophyticus* *S. xylosus*	Samelis et al., 1998
	N.S.	*S. saprophyticus* *S. carnosus* *S. xylosus* *K. varians*	Papamanoli et al., 2002
	N.S.	*L. sakei* *L. curvatus* *L. plantarum* *L. paracasei* subsp. *paracasei* *L. buchneri* *W. viridescens* *W. pseudomesenteroides*	Papamanoli et al., 2003
	Dry sausages	*L. plantarum* *L. plantarum/pentosus* *S. saprophyticus* *S. xylosus* *S. simulans*	Drosinos et al., 2005
France	N.S.	*S. xylosus* *S. carnosus* *S. saprophyticus* *S. epidermidis*	Montel et al., 1993
Italy	N.S.	*L. sakei* *L. curvatus* *L. plantarum*	Torriani et al., 1990
	"Salame friulano"	*S. xylosus* *S. intermedius* *S. simulans*	Comi et al., 1992
	"Soppressata molisana"	*S. xylosus* *S. simulans* *S. equorum* *S. kloosii* *K. kristinae* *K. roseus* *K. varians*	Coppola et al., 1997
	"Soppressata molisana"	*L. sakei* *L. plantarum* *L. curvatus*	Coppola et al., 1998
	N.S.	*S. xylosus* *S. sciuri*	Rebecchi et al., 1998
	"Salame tipo Napoli"	*L. sakei* *S. xylosus* *S. saprophyticus*	Coppola et al., 2000
	"Soppressata" and "salsiccia"	*L. sakei* *Lc. carnosum* *Lc. gelidum* *W. paramesenteroides*	Parente et al., 2001
	"Salame tipo Napoli", "sopressata di	*S. xylosus* *S. saprophyticus*	Mauriello et al., 2004

(continued on next page)

Table 1 (*continued*)

Country	Traditional product	Bacterial species	Reference
	Ricigliano", "soppressata di Gioi"	*S. warneri* *S. lentus*	
Spain	N.S.	*L. sakei* *L. curvatus*	Sanz et al., 1988
	N.S.	*S. capitis* *S. epidermidis* *S. simulans*	del Carmen de la Rosa et al., 1990
	N.S.	*L. curvatus* *L. plantarum*	Hugas et al., 1993
	"Chorizo"	*L. sakei* *L. curvatus* *Pediococcus* sp.	Santos et al., 1998
	"Chorizo"	*S. xylosus*	Garcia-Varona et al., 2000

The species are ordered on the basis of their prevalence in the product studied. Abbreviations. N.S., not specified.

focused only on one group of bacteria. In the case of Rebecchi et al. (1998), isolation of LAB and CNC was performed but biochemical tests were applied only on CNC, while LAB were identified by molecular methods. From Table 1, it can be concluded that some species of LAB and CNC are widespread and isolated from several types of fermented sausages produced in different countries.

Concerning the LAB, it is apparent that *L. sakei* and *L. curvatus* are the most adapted species of *Lactobacillus* spp. to meat fermentations. Among the studies reported here and considering the LAB populations, at least one and in some cases both of the species were isolated. As a matter of fact, only Samelis et al. (1998), Coppola et al. (1998) and Coppola et al. (2000) did not isolate *L. curvatus*. Other species of *Lactobacillus* were also identified. It seems that *Lactobacillus plantarum* is another important species that can participate to sausage fermentations, but its frequency of isolation is not as high as for the other two species mentioned above. Samelis et al. (1994) and Papamanoli et al. (2003) described the isolation and identification of other members of *Lactobacillus* (i.e. *Lactobacillus farciminis*, *Lactobacillus coryniformis*, *Lactobacillus casei* subsp. *pseudoplantarum*, *Lactobacillus paracasei* subsp. *paracasei* and *Lactobacillus buchneri*) that were never found in fermented sausages apart from the Greek salami. Moreover, representative of the genus *Weissella*, originally proposed by Collins et al. (1993), were identified from these Greek products, leading to the hypothesis that they are country specific. Among all the studies presented in Table 1, only a few presented sporadic isolation of *Weissella* spp. (Parente et al., 2001). However, it should be mentioned that members of *Weissella* are undesirable contaminants, due to their hetero-fermentative metabolism.

It is interesting to point out that only in the case of Santos et al. (1998) wild strains of *Pediococcus* spp. were found during the fermentation process. *Pediococcus* spp. are among the most common starter cultures used in fermented meat products in the USA, while in Europe, *Lactobacillus* spp. are more often used (Bacus and Brown, 1981). This fact is raising the issue of whether or not *Pediococcus* spp. should be used as starter culture for the production of traditional products in Europe. As a matter of fact, it has been demonstrated that starter cultures isolated from one product have failed to lead fermentation when used to produce a different product (Marchesini et al., 1992).

Taking into consideration the CNC microflora, the species always isolated, independently of the country of production, is *S. xylosus*. It often dominates the CNC populations and this is justifying its wide use as starter culture. Another species, among *Staphylococcus* spp., that was frequently isolated from the fermentation process is *Staphylococcus saprophyticus* isolated from Greek sausages (Samelis et al., 1998; Papamanoli et al., 2002), and from Naples-type salami (Coppola et al., 2000; Mauriello et al., 2004). *Staphylococcus carnosus*, *Staphylococcus simulans*, *Staphylococcus equorum*, *Staphylococcus kloosii*, *Staphylococcus sciuri*, *Staphylococcus warneri* and *Staphylococcus lentus*, as well as *Kocuria* spp., were identified at lesser extend. However, in freshly prepared sausages, *Kocuria* spp. were found to be the dominant population (Comi et al., 1992; Rodriguez et al., 1994). It is worth mentioning that, by applying direct culture-independent methods in fresh sausages, *Kocuria* spp. were never detected from the day of production to the 10th day of storage at 4°C (Cocolin et al., 2004).

4.2. Identification by molecular methods

For the purpose of identification of isolates by molecular methods, the gene more often targeted is the 16S rRNA. It possesses some key attributes that make it suitable for bacterial identification. In particular, it is common in all bacteria, it possess variable and conserved regions that can be used for differentiation purposes and lastly, it functions as an evolutionary clock, allowing for conclusions to be drawn regarding phylogeny (Woese, 1987; Collins et al., 1991; Gürtler and Stanisich, 1996; Nour, 1998). Moreover, a large database of sequences is already available. Despite the large number of molecular techniques developed for the identification of LAB and CNC isolated from fermented sausages, only some were applied in order to define the microflora of these products. The studies that exploited molecular methods for this purpose are reported in Table 2. They should be included in the category of culture-dependent methods, since the application is on strains traditionally isolated (Fig. 1). The first paper applying molecular methods for the identification of LAB was published in 1998 by Rebecchi et al. The authors used RAPD analysis to group the isolated LAB that were subsequently subjected to the 16S rRNA gene sequencing. The authors reported that the isolates could be grouped in a very limited number of groups with the same RAPD pattern, and after sequencing they resulted to belong to *L. sakei* and *L. plantarum*. The RAPD-PCR approach was also used by Andrigetto et al. (2001) that identified *L. sakei* and *L. curvatus* from traditional salami produced in the Veneto region, Italy. Versatile methods, recently extensively used for strain identification, are DGGE and TGGE. Since the separation between strains is based on differential migrations, these techniques can be used for screening and grouping the isolates, thereby reducing the number of cultures to identify by 16S rRNA gene sequencing. This approach was used to identify LAB during

production of Italian, Greek and Hungarian fermented sausages (Cocolin et al., 2000; Comi et al., 2005; Rantsiou et al., 2005a). It is interesting to notice that the application of PCR-DGGE and 16S rRNA gene sequencing allow the identification of a large number of strains in a quick and fast way. PCR-DGGE was also exploited to identify CNC from Italian sausages (Cocolin et al., 2001a). 16S-23S rRNA genes intergenic PCR and species-specific PCR were used as well, mainly for the identification of CNC (Rossi et al., 2001; Blaiotta et al., 2003b, 2004; Rantsiou et al., 2005b). In this last case, some strains could not be definitively identified (Blaiotta et al., 2004) (Table 2).

The picture of the fermented sausage microflora that can be observed with the application of molecular methods does not disagree with the one produced using biochemical identification. Once more the predominance of *L. sakei* and *L. curvatus*, and of *S. xylosus*, is emerging. As already described for the biochemical tests, also in this case other lactobacilli are identified, but their numbers were found to be significantly lower than *L. sakei* and *L. curvatus*. *Leuconostoc* and *Weissella* spp. were identified at lesser extend, underlining possible pitfalls in their identification by traditional methods, or more simply their low presence in the type of sausages studied. Also in the case of CNC, apart from *S. xylosus* other species were described. *S. carnosus*, *S. simulans*, *Staphylococcus condimenti*, *Staphylococcus pulvereri/vitulis*, *S. equorum*, and *S. saprophyticus* were identified among the CNC isolates, and it is interesting to notice that some species were identified only when molecular methods were applied. *Kocuria* spp. were identified only in "salame friulano" (Cocolin et al., 2001a).

5. Culture-independent methods

The papers describing the application of culture-independent methods in the definition of the microflora in fermented sausages are reported in Table 2. As described above, the culture-independent methods are based on a direct extraction of DNA and/or RNA from the sample under investigation. The purified nucleic acids are subsequently subjected to molecular methods that are able to profile the bacterial populations present. The approaches that can be considered are either based on the use of molecular probes, or the use of PCR by itself or coupled with other techniques. Despite the fact that specific probes have been developed for the identification of lactobacilli commonly isolated from meat (Hertel et al., 1991; Nissen and Dainty, 1995), there are no papers available on the application of these probes to target specific LAB directly in the sausages during fermentation. The available data refer only to the rapid identification of isolated strains.

For the purpose of the direct profiling of the microflora in fermented sausages the main protocols exploited were species-specific PCR and the PCR-DGGE method. It is important to underline that the direct approaches have only recently been applied to sausage fermentations. Studies so far available on the direct profiling of sausage microflora have been performed on Italian and Spanish sausages (Cocolin et al., 2001c; Aymerich et al., 2003; Rantsiou et al., 2005c).

Table 2
LAB and CNC isolated from naturally fermented sausages from different countries and identified by culture-dependent and -independent molecular methods

Country	Traditional product	Bacterial species[a]	Identification method	Reference
Culture-dependent methods:				
Greece	NS	*L. curvatus* *L. sakei* *L. plantarum* *L. casei/paracasei*[a] *L. paraplantarum*	PCR-DGGE and 16S rRNA gene sequencing	Rantsiou et al., 2005a
Hungary	N.S.	*L. sakei* *L. curvatus* *W. viridescens* *W. paramesenteroides/hellenica*[a] *Leuconostoc mesenteroides*	PCR-DGGE and 16S rRNA gene sequencing	Rantsiou et al., 2005a
Italy	N.S.	*L. sakei*	RAPD analysis and 16S rRNA gene sequencing	Rebecchi et al., 1998
	"Salame friulano"	*L. plantarum* *L. sakei* *L. casei* *L. curvatus* *L. alimentarius*	PCR-TGGE	Cocolin et al., 2000
	"Salame friulano"	S. xylosus *S. carnosus* *S. simulans* *K. varians*	PCR-DGGE	Cocolin et al., 2001a
	"Soppressata", "salame tradizionale"	*L. sakei* *L. curvatus*	RAPD-PCR	Andrigetto et al., 2001
	"Salsiccia sotto sugna"	*S. xylosus* *S. condimenti*	16S-23S rRNA genes intergenic region PCR	Rossi et al., 2001
	N.S.	*S. xylosus*	16S rRNA gene sequencing	Blaiotta et al., 2004
		S. pulvereri/vitulus[a]	Intergenic spacer region PCR	
		S. equorum *S. saprophyticus*	PCR-DGGE Species-specific PCR	
	"Salame friulano"	*L. sakei* *L. curvatus* *L. plantarum* *L. paraplantarum* *W. paramesenteroides/hellenica*[a]	PCR-DGGE and 16S rRNA gene sequencing	Rantsiou et al., 2005a
	"Salame friulano"	*L. sakei* *L. curvatus* *L. plantarum* *L. paraplantarum*	PCR-DGGE and 16S rRNA gene sequencing	Comi et al., 2005
Culture-independent methods:				
Italy	"Salame friulano"	*L. sakei* *L. curvatus* *S. xylosus*	16S rRNA gene PCR-DGGE	Cocolin et al., 2001c

(continued on next page)

Table 2 (*continued*)

Country	Traditional product	Bacterial species [a]	Identification method	Reference
		S. intermedius *S. carnosus* *S. lentus* *S. pulvereri* *L. plantarum*		
	"Salame friulano"	*L. sakei* *L. curvatus* *S. xylosus* *L. plantarum*	*rpoB* gene PCR-DGGE	Rantsiou et al., 2004
	"Salame friulano"	*L. sakei* *L. curvatus* *L. paracasei* *S. xylosus* *S. sciuri/pulvereri* [a] *S. equorum/ succinus* [a] *L. garvieae* *S. intermedius* *M. caseolyticus*	16S rRNA gene PCR-DGGE	Rantsiou et al., 2005c
Spain	"Fuet" [b]	*L. sakei* *L. curvatus* *L. plantarum* *S. xylosus*	Direct species-specific PCR	Aymerich et al., 2003
	"Chorizo" [b]	*L. sakei* *L. plantarum* *L. curvatus* *S. xylosus*	Direct species-specific PCR	Aymerich et al., 2003

The species are ordered on the basis of their prevalence in the product studied.
[a] The method used did not allow a definitive identification.
[b] Results obtained after an enrichment step at 30 °C for 24 h in MRS broth and in mannitol salt broth for LAB and CNC, respectively.

Aymerich et al. (2003) described the use of several species specific primers to identify without traditional isolation and identification, LAB and CNC members in "chorizo" and "fuet", two traditional fermented sausages produced in Spain. The investigation focused on products at the end of maturation, and the experimental approach consisted of the amplification of DNA extracted from the sample, either subjected to an enrichment step or not. Six species of LAB and six species of CNC were targeted with specific primers. In particular, *L. sakei*, *L. curvatus*, *L. plantarum*, *Enterococcus faecium*, *Lactococcus lactis*, *Pediococcus acidilactici*, *S. carnosus*, *S. warneri*, *Kocuria varians*, *S. xylosus*, *S. simulans* and *Staphylococcus epidermidis* were considered. Authors concluded that *L. sakei* and *S. xylosus* can be considered the predominant species in slightly fermented sausages produced in Spain. Also *L. plantarum*, detected in 100% of "chorizos" and in only 50% of the "fuets" should be considered an important agent of Spanish sausage fermentation. This difference in the detection should be explained by the different technology used for the production of the two types of sausages studied.

The use of the enrichment can be considered to introduce biases in the results obtained on the profiling of the microflora, in fact some populations can take over and inhibit the growth of numerically less important species. However, the use of selective or elective media in samples that will be subjected to DNA extraction and PCR, is a common practice in the field of food microbiology because it helps to overcome problems related with the PCR method. More specifically, with an enrichment step, target cells are increasing in the number making their detection easier, only alive cells are amplified, and, lastly, by diluting the sample in liquid enrichment media, also PCR inhibitors are diluted.

The disadvantage of a method that is based on species specific probes for hybridisation or species specific primers for amplification is that, unavoidably, the number of species that may be detected is limited and it is lower or equal to the number of probes or sets of primers. These methods are applicable if we are interested in the dynamics of specific organisms. But if we are interested in obtaining a more complete picture of the fermentation microflora and its evolution with time an alternative technique is required.

The most suitable technique for the profiling of the microflora present in a specific environment should be able to detect simultaneously all the bacterial species present. This goal can be almost completely achieved with the application of the PCR-DGGE technique. Additionally, DGGE supports the species identification of community members because the amplification products, after they have been separated by DGGE, they can be recovered from the gels and sequenced (Cocolin et al., 2001c, 2004; Mills et al., 2002).

The PCR-DGGE method has been applied in our laboratory to profile the dynamic change during sausage fermentation, with the goal of understanding the population successions and to investigate which species are metabolically active during the process (Cocolin et al., 2001c; Rantsiou et al., 2005c). In these studies the V1 region of the 16S rRNA gene was selected for the amplification purpose. It has been recently reported that the use of V1 region alone in DGGE analysis should be used carefully, because of its limited length (Yu and Morrison, 2004). However, in the first study performed on DGGE profiling of fermented sausage microflora (Cocolin et al., 2001c), it was concluded that only PCR products of the V1 region allow the differentiation between LAB and CNC involved in the transformation process. Products produced from the V3, V6–V8 and V9 regions of the 16S rRNA genes resulted always in co-migrations of control species of LAB and CNC used for the optimisation of the protocol (Cocolin et al., 2001c). In this study, both DNA and RNA were sampled directly in order to determine the levels of expression of the 16S rRNA gene of the most prominent bacteria, which may reflect their contributions to the fermentation process. The main difference detected by sampling RNA rather than DNA was the presence of natural meat contaminants, such as *Brochotrix thermosphacta*, *Enterococcus* sp., *Leuconostoc mesenteroides* and *Brevibacillus* sp., which were not present after the third day. *Staphylococcus* species were found only in the meat mixture before 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. *L. sakei* and *L. curvatus* were the two species of LAB strongly present at both DNA and RNA level from the 3rd day of fermentation. One band, at DNA level, was identified as *L. plantarum*, but it was probably generated from dead cells present in the meat, since no

L. plantarum cells were isolated during fermentation and no specific signal was detected in the RNA amplicons.

Rantsiou et al. (2005c) in the last year completed a study in which a comparison of the microflora, determined by PCR-DGGE, of three traditional fermented sausages produced in three plants of the Northeastern part of Italy, was performed. The bacterial ecology was mainly characterised by the stable presence of *L. curvatus* and *L. sakei*, but also *L. paracasei* was repeatedly detected. Important evidence was the presence of *Lactococcus garvieae*, which clearly contributed in two fermentations. Several members of *Staphylococcus* were also detected. Regarding other bacterial groups *Bacillus* sp., *Ruminococcus* sp. and *Macrococcus caseolyticus* were also identified at the beginning of the transformations. In addition, yeast species belonging to *Debaryomyces hansenii*, several *Candida* species and *Willopsis saturnus* were observed in the DGGE gels. Finally, cluster analysis of the bacterial and yeast DGGE profiles highlighted the uniqueness of the fermentation processes studied.

A high level of information could be obtained by the application of molecular methods. The importance of species commonly recognised as responsible for sausage fermentation (i.e. *L. sakei* and *L. curvatus*) was confirmed. In addition, for the first time, evidence was obtained that, strains belonging to *L. garvieae* and *M. caseolyticus*, that are commonly isolated from dairy products, may also be involved in sausage fermentation. Since milk powder and/or lactose are commonly used in the production of fermented sausages, it can be assumed that these microorganisms come from these sources. However, the sausages studied by Rantsiou et al. (2005c) did not contain these ingredients in their formula.

The application of 16S rRNA gene PCR-DGGE analysis is not, however, free of problems. One hurdle to overcome is the sensitivity of the method for the detection of low number populations. Cocolin et al. (2001c) defined that in mixed populations, individual members could be identified by PCR-DGGE of the V1 region when the concentrations are more than 10^4 cfu/g, which allows detection of species at a threshold level during fermentation. This may not be true when other primers are applied in the PCR amplification step. As a consequence, special attention should be dedicated to this issue to carefully profile the microflora present in specific ecosystems. A second problem, related to the technique when rRNA amplicons are analysed by DGGE, is the presence of multiple copies of the ribosomal genes. Due to its heterogeneity, several bands per species can be seen in high-resolution PCR-DGGE analysis. The amplified fragments will therefore appear as several bands on a DGGE gel, rather than a single band that would allow precise species identification.

A possible solution to the heterogeneity of the 16S rRNA gene was given by Dahllof et al. (2000), which proposed the *rpoB* gene, coding for the β subunit of the RNA polymerase, as a target for PCR-DGGE. The approach resulted to be convenient for environmental samples (Dahllof et al., 2000), and it was further applied to follow the fermentation of sausages (Rantsiou et al., 2004). In this study, the contribution of *L. sakei*, *L. curvatus* and *S. xylosus* was again pointed out. Interestingly, a band belonging to *L. plantarum* was constantly present after the 10th day of fermentation.

Lastly, the incomplete extension of the GC clamp during PCR amplification, which may result in artifactual double bands in DGGE analysis, often complicates the interpretation of the profiles. This issue was recently addressed by Janse et al. (2004) and it was proposed that it can be eliminated by increasing the time of the final extension step during CPR.

6. Conclusions

Sausage fermentation is a complex system of events in which microorganisms represent key agents for the production of specific compounds and enzymes that allow the transformation of raw meat in a product with new physico-chemical and sensory characteristics. In this process the quality of the meat and of the ingredients (salt, spices, nitrate and nitrite), the microorganisms operating the transformations (added as a starter or naturally present) and the fermentation and ripening conditions are fundamental parameters to control in order to obtain final products with the desired organoleptic profile.

It is widely accepted that the contribution of microorganisms, in particular LAB and CNC, is essential to achieve specific sensory characteristics during sausage fermentations. For this reason, the study of the microflora is an important aspect to take into account.

Considering the data available on the microflora in fermented sausages, it can be concluded that there is a good correlation between the results obtained with traditional methods and the ones achieved with molecular methods of identification. With both approaches it has been repeatedly highlighted that species belonging to *L. sakei*, *L. curvatus* and *S. xylosus* are the best adapted to the sausage fermentation ecosystem, thereby dominating the microflora present. However, small differences can be observed in cases of traditional products coming from a specific country. As an example, using traditional methods researchers from Greece defined that dry salami were characterised by the presence of *Weissella* members, such as *Weissella viridescens*, *Weissella hellenica* and *Weissella paramensenteroides* (Samelis et al., 1994; Papamanoli et al., 2003). This evidence was not confirmed by molecular methods, in fact in one study performed using PCR-DGGE followed by 16S rRNA gene sequencing, it was determined the absence of these species in sausages produced in Greece (Rantsiou et al., 2005a). The different results obtained can be explained very simply considering that the studies were performed on different samples, so it is possible that in the last case sausages did not contain *Weissella* spp., but it is acceptable to recognise a probable overestimation of this genera with traditional methods. This question could be addressed only if a comparative study on the microflora of Greek fermented sausages was available.

With the development of molecular methods of detection and identification new frontiers could be reached. Moreover, the possibility to profile these populations directly in the sample, without the need of traditional isolation and identification, allows scientists for the first time to investigate the microbial interrelations in situ.

Considering the culture-independent methods, once more the capability of *L. sakei*, *L. curvatus* and *S. xylosus* to dominate the fermentation process is obvious, but new interesting information is also obtained. As a matter of fact, it seems that other species of LAB and CNC are active in the transformation process as well. *L. paracasei* was identified by the means of direct PCR-DGGE method in Italian sausages. Moreover, also *L. garvieae* seems to contribute to the fermentation. In the group of CNC, *S. equorum* and *S. succinus*, although isolated in sausages at low percentages by traditional methods, they were often detected by direct methods, therefore they should be considered important.

Future developments in the field of sausage fermentation can be represented by studies on strain biodiversity with the final goal to try to explain the extreme diversity of products. Sausages, produced in different countries but also within the same country, are characterised by different organoleptic profiles and sensory characteristics, and this aspect can be only partially explained considering the actual ingredients used. In our opinion the microflora plays a major role by this point of view, and it is possible that the differences in the sensory characteristics of the products are due to the presence of different strains. This aspect becomes even more interesting considering that in the fermented sausages studied so far, it was always defined that the main species found were the same among the different products. A possible explanation could be the presence within the species of specific strains unique for a specific product. It has been defined, for example, that within the species *L. sakei* and *L. curvatus* there are populations that can be differentiated based on their provenience (Rantsiou et al., 2005a). Moreover, a study performed on about 350 LAB strains, isolated from three plants of continental Greece, highlighted the presence of plant-specific populations (Rantsiou et al., unpublished results). Little is known about the intra-species differences of LAB strains involved in the sausage fermentations. However, the plant-specific clusters found are highlighting that the production conditions (temperature, moisture and ingredients), that differ from plant to plant, are possibly able to determine the selection of a specific population, and more precisely of a specific strain within the species that will take over during transformation, thereby characterising the final product produced in the specific plant. To us this is an important aspect to consider to completely understand the fermentation process, and it should 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, are crucial since these are the parameters that will have a great impact on the organoleptic and sensorial characteristics of the final product.

Acknowledgements

This review was developed within the frame of the projects funded by the Ministry of University, Rome, Italy, action PRIN (ex 40%) and by the E. C. within the framework of the specific research and technological development program "Confirming the International Role of the Community Research", (Contract n. ICA4-CT-2002-10037). Authors would like to express their gratitude to Prof. Giuseppe Comi and Prof. Apostolos Rantsios for their critical and careful review of this manuscript.

References

Akane, A., Matsubara, K., Nakamura, H., Takahashi, S., Kimura, K., 1994. Identification of the heme compound copurified with deoxyribonucleic acid (DNA) from blood stains, a major inhibition of polymerase chain reaction (PCR) amplification. Journal of Forensic Science 39, 362–372.

American Public Health Association, 1966. Recommended Methods for the Microbiological Examination of Foods, 2nd Ed. APHA Inc., New York.

Andrigetto, C., Zampese, L., Lombardi, A., 2001. RAPD-PCR characterization of lactobacilli isolated from artisanal meat plants and traditional fermented sausages of Veneto region (Italy). Letters in Applied Microbiology 33, 26–30.

Aymerich, T., Martin, B., Garriga, M., Hugas, M., 2003. Microbial quality and direct PCR identification of lactic acid bacteria and nonpathogenic staphylococci from artisanal low-acid sausages. Applied and Environmental Microbiology 69, 4583–4594.

Bacus, J.N., 1986. Fermented meat and poultry products. In: Pearson, A.M.D. (Ed.), Advances in Meat and Poultry Microbiology. Macmillan, London, pp. 123–164.

Bacus, J.N., Brown, W.L., 1981. Use of microbial cultures: meat products. Food Technology 35, 74–83.

ben Omar, N., Ampe, F., 2000. Microbial community dynamics during production of the Mexican fermented maize dough pozol. Applied and Environmental Microbiology 66, 3664–3673.

Berthier, F., Ehrlich, S.D., 1998. Rapid species identification within two groups of closely related lactobacilli using PCR primers that target the 16S/23S rRNA spacer region. FEMS Microbiology Letters 161, 97–106.

Berthier, F., Ehrlich, S.D., 1999. Genetic diversity within *Lactobacillus sakei* and *Lactobacillus curvatus* and design of PCR primers for its detection using randomly amplified polymorphic DNA. International Journal of Systematic Bacteriology 49, 997–1007.

Blaiotta, G., Pennacchia, C., Ercolini, D., Moschetti, G., Villani, F., 2003a. Combining denaturing gradient gel electrophoresis of 16S rDNA V3 region and 16S-23S rDNA spacer region polymorphism analyses for the identification of staphylococci from Italian fermented sausages. Systematic and Applied Microbiology 26, 423–433.

Blaiotta, G., Pennacchia, C., Parente, E., Villani, F., 2003b. Design and evaluation of specific PCR primers for rapid and reliable identification of *Staphylococcus xylosus* strains isolated from dry fermented sausages. Systematic and Applied Microbiology 26, 601–610.

Blaiotta, G., Pennacchia, C., Villani, F., Ricciardi, A., Tofalo, R., Parente, E., 2004. Diversity and dynamics of communities of coagulase-negative staphylococci in traditional fermented sausages. Journal of Applied Microbiology 97, 271–284.

Buffone, G.J., Demmler, G.J., Schimbor, C.M., Greer, J., 1991. Improved amplification of cytomegalovirus DNA from urine after purification of DNA with glass beads. Clinical Chemistry 37, 1945–1949.

Charteris, W.P., Kelly, P.M., Morelli, L., Collins, J.K., 1997. Selective detection, enumeration and identification of potentially probiotic *Lactobacillus* and *Bifidobacterium* species in mixed bacterial populations. International Journal of Food Microbiology 35, 1–27.

Cocolin, L., Mills, D.A., 2003. Wine yeast inhibition by sulfur dioxide: a comparison of culture-dependent and independent methods. American Journal of Enology and Viticulture 54, 125–130.

Cocolin, L., Manzano, M., Cantoni, C., Comi, G., 2000. Development of a rapid method for the identification of *Lactobacillus* spp. isolated from naturally fermented Italian sausage using a polymerase chain reaction–temperature gradient gel electrophoresis. Letters in Applied Microbiology 2000, 126–129.

Cocolin, L., Manzano, M., Aggio, D., Cantoni, C., Comi, G., 2001a. A novel polymerase chain reaction (PCR)–denaturing gradient gel electrophoresis (DGGE) for the identification of Micrococcaceae strains involved in meat fermentations. Its application to naturally fermented Italian sausages. Meat Science 58, 59–64.

Cocolin, L., Heisey, A., Mills, D.A., 2001b. Direct identification of the indigenous yeasts in commercial wine fermentations. American Journal of Enology and Viticulture 52, 49–53.

Cocolin, L., Manzano, M., Cantoni, C., Comi, G., 2001c. Denaturing gradient gel electrophoresis analysis of the 16S rRNA gene V1 region to monitor dynamic changes in the bacterial population during fermentation of Italian sausages. Applied and Environmental Microbiology 67, 5113–5121.

Cocolin, L., Aggio, D., Manzano, M., Cantoni, C., Comi, G., 2002a. An application of PCR-DGGE analysis to profile the yeast populations in raw milk. International Dairy Journal 12, 407–411.

Cocolin, L., Rantsiou, K., Iacumin, L., Cantoni, C., Comi, G., 2002b. Direct identification in food samples of *Listeria* spp. and *Listeria monocytogenes* by molecular methods. Applied and Environmental Microbiology 68, 6273–6282.

Cocolin, L., Rantsiou, K., Iacumin, L., Urso, R., Cantoni, C., Comi, G., 2004. Study of the ecology of fresh sausages and characterization of populations of lactic acid bacteria by molecular methods. Applied and Environmental Microbiology 70, 1883–1894.

Cocolin, L., Comi, G., 2005. Use of a culture independent molecular method for the direct detection and identification of *Yersinia* spp. in food. International Journal of Food Microbiology 105, 71–82.

Collins, M.D., Rodriguez, U., Ash, C., Aguirre, M., Farrow, J.E., Martinez-murcia, A., Philips, B.A., Williams, A.M., Wallbanks, S., 1991. Phylogenetic analysis of the genus *Lactobacillus* and related lactic acid bacteria as determined by reverse-transcriptase sequencing of 16S ribosomal-RNA. FEMS Microbiology Letters 77, 5–12.

Collins, M.D., Samelis, J., Metaxopoulos, J., Wallbanks, S., 1993. Taxonomic studies on some leuconostoc-like organisms from fermented sausages: description of the new genus *Weissella* for the *Leuconostoc paramesenteroides* group of species. Journal of Applied Bacteriology 75, 595–603.

Comi, G., Citterio, B., Manzano, M., Cantoni, C., 1992. Evaluation and characterisation of Micrococcaceae strains in Italian dry fermented sausages. Fleischwirtschaft 72, 1679–1683.

Comi, G., Urso, R., Iacumin, L., Rantsiou, K., Cattaneo, P., Cantoni, C., Cocolin, L., 2005. Characterisation of naturally fermented sausages produced in the North East of Italy. Meat Science 69, 381–392.

Coppola, R., Iorizzo, M., Saotta, R., Sorrentino, E., Grazia, L., 1997. Characterization of micrococci and staphylococci isolated from soppressata molisana, a southern Italy fermented sausage. Food Microbiology 14, 47–53.

Coppola, R., Giagnacovo, B., Iorizzo, M., Grazia, L., 1998. Characterization of lactobacilli involved in the ripening of soppressata molisana, a typical southern Italy fermented sausage. Food Microbiology 15, 347–353.

Coppola, S., Mauriello, G., Aponte, M., Moschetti, G., Villani, F., 2000. Microbial succession during ripening of Naples-type salami, a southern Italian fermented sausage. Meat Science 56, 321–329.

Corbiere Morot-Bizot, S., Talon, R., Leroy, S., 2004. Development of a multiplex PCR for the identification of *Staphylococcus* genus and four staphylococcal species isolated from food. Journal of Applied Microbiology 97, 1087–1094.

Dahllof, I., Baillie, H., Kjelleberg, S., 2000. *rpo*B-based microbial community analysis avoids limitations inherent in 16S rRNA gene intraspecific heterogeneity. Applied and Environmental Microbiology 66, 3376–3380.

del Carmen de la Rosa, M., Mohino, M.R., Mohino, M., Mosso, M.A., 1990. Characteristics of micrococci and staphylococci isolated from semi-preserved meat products. Food Microbiology 7, 207–215.

de Man, J.C., Rogosa, M., Sharpe, M.E., 1960. A medium for the cultivation of lactobacilli. Journal of Applied Bacteriology 23, 130–138.

Demeyer, D.I., 1982. Stoichiometry of dry sausage fermentation. Antonie Leeuwenhoek 48, 414–416.

Demeyer, D.I., Verplaetse, A., Gistelink, M., 1986. Fermentation of meat: an integrated process. Belgian Journal of Food Chemistry and Biotechnology 41, 131–140.

Di Maria, S., Basso, A.L., Santoro, E., Grazia, L., Coppola, R., 2002. Monitoring of *Staphylococcus xylosus* DSM 20266 added as starter during fermentation and ripening of soppressata molisana, a typical Italian sausage. Journal of Applied Microbiology 92, 158–164.

Drosinos, E.H., Mataragas, M., Xiraphi, N., Moschonas, G., Gaitis, F., Metaxopoulos, J., 2005. Characterization of the microbial flora from traditional Greek fermented sausage. Meat Science 69, 307–317.

Ercolini, D., 2004. PCR-DGGE fingerprinting: novel strategies for detection of microbes in food. Journal of Microbiological Methods 56, 297–314.

Ercolini, D., Moschetti, G., Blaiotta, G., Coppola, S., 2001a. Behavior of variable V3 region from 16S rDNA of important lactic acid bacteria in denaturing gradient gel electrophoresis. Current Microbiology 42, 199–202.

Ercolini, D., Moschetti, G., Blaiotta, G., Coppola, S., 2001b. The potential of a polyphasic PCR-DGGE approach in evaluating microbial diversity of natural whey cultures from water-buffalo Mozzarella cheese production: bias of "culture dependent" and "culture independent" approaches. Systematic and Applied Microbiology 24, 610–617.

Ercolini, D., Hill, P.J., Dodd, E.R., 2003. Bacterial community structure and location in Stilton cheese. Applied and Environmental Microbiology 69, 3540–3548.

Ercolini, D., Mauriello, G., Blaiotta, G., Moschetti, G., Coppola, S., 2004. PCR-DGGE fingerprints of microbial succession during a manufacture of traditional water buffalo Mozzarella cheese. Journal of Applied Microbiology 96, 263–270.

Fadda, S., Vignolo, G., Holgado, A.P.R., Oliver, G., 1998. Proteolytic activity of *Lactobacillus* strains isolated from dry-fermented sausages on muscle sarcoplasmic proteins. Meat Science 49, 11–18.

Fadda, S., Sanz, Y., Vignolo, G., Aristoy, M.-C., Oliver, G., Toldra', F., 1999a. Characterization of muscle sarcoplasmatic and myofibrillar protein hydrolysis caused by *Lactobacillus plantarum*. Applied and Environmental Microbiology 65, 3540–3546.

Fadda, S., Sanz, Y., Vignolo, G., Aristoy, M.-C., Oliver, G., Toldra', F., 1999b. Hydrolysis of pork muscle sarcoplasmatic proteins by *Lactobacillus curvatus* and *Lactobacillus sake*. Applied and Environmental Microbiology 65, 578–584.

Fadda, S., Oliver, G., Vignolo, G., 2002. Protein degradation by *Lactobacillus plantarum* and *Lactobacillus casei* in a sausage model system. Journal of Food Science 67, 1179–1183.

Garcia-Varona, M., Santos, E.M., Jaime, I., Rovira, J., 2000. Characterization of Micrococcaceae isolated from different varieties of chorizo. International Journal of Food Microbiology 54, 189–195.

Gevers, D., Huys, G., Swings, J., 2001. Applicability of rep-PCR fingerprinting for identification of *Lactobacillus* species. FEMS Microbiology Letters 205, 31–36.

Gürtler, V., Stanisich, V.A., 1996. New approaches to typing and identification of bacteria using the 16S-23S rRNA spacer region. Microbiology 141, 1255–1265.

Hammes, W.P., Knauf, H.J., 1994. Starters in processing of meat products. Meat Science 36, 155–168.

Hammes, W.P., Bantleon, A., Min, S., 1990. Lactic acid bacteria in meat fermentation. FEMS Microbiology Reviews 87, 165–174.

Head, I.M., Saunders, J.R., Pickup, R.W., 1998. Microbial evolution, diversity and ecology: a decade of ribosomal RNA analysis of uncultivated microorganisms. Microbial Ecology 35, 1–21.

Hertel, C., Ludwig, W., Obst, M., Vogel, R.F., Hammes, W.P., Schleifer, K. H., 1991. 23S rRNA-targeted oligonucleotide probes for the rapid identification of meat lactobacilli. Systematic and Applied Microbiology 14, 173–177.

Heuer, H., Smalla, K., 1997. Application of denaturing gradient gel electrophoresis (DGGE) and temperature gel electrophoresis (TGGE) for studying soil microbial communities. In: van Elsas, J.D., Wellington, E.M.H., Trevors, J.T. (Eds.), Modern Soil Microbiology. Marcel Dekken, New York, pp. 353–373.

Hugas, M., Garriga, M., Aymerich, T., Monfort, J.M., 1993. Biochemical characterization of lactobacilli isolated from dry fermented sausages. International Journal of Food Microbiology 18, 107–113.

Hugenholtz, P., Pace, N.R., 1996. Identifying microbial diversity in the natural environment: a molecular phylogenetic approach. Trends in Biotechnology 14, 90–97.

Hugenholtz, P., Goebel, B.M., Pace, N.R., 1998. Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. Journal of Bacteriology 180, 4765–4774.

Janse, I., Bok, J., Zwart, G., 2004. A simple remedy against artifactual double bands in denaturing gradient gel electrophoresis. Journal of Microbiological Methods 57, 279–281.

Lee, J., Jang, J., Kim, B., Kim, J., Jeong, G., Han, H., 2004. Identification of *Lactobacillus sakei* and *Lactobacillus curvatus* by multiplex PCR-based restriction enzyme analysis. Journal of Microbiological Methods 59, 1–6.

Lerche, M., Reuter, G., 1960. A contribution to the method of isolation and differentiation of aerobic "lactobacilli" (genus *Lactobacillus* Beijerinck). Arch for Hygiene und Bakteriologie 179, 354–370.

Lücke, F.K., 1974. Fermented sausages. In: Wood, B.J.B. (Ed.), Microbiology of Fermented Foods. Applied Science Publishers, London, pp. 41–49.

Lücke, F.K., 2000. Utilization of microbes to process and preserve meat. Meat Science 56, 105–115.

Marchesini, B., Bruttin, A., Romailler, N., Moreton, R.S., Stucchi, C., Sozzi, T., 1992. Microbiological events during commercial meat fermentations. Journal of Applied Bacteriology 73, 203–209.

Mauriello, G., Casaburi, A., Blaiotta, G., Villani, F., 2004. Isolation and technological properties of coagulase negative staphylococci from fermented sausages of southern Italy. Meat Science 67, 149–158.

Meroth, C.B., Hammes, W.P., Hertel, C., 2003a. Identification and population dynamics of yeasts in sourdough fermentation processes by PCR-denaturing gradient gel electrophoresis. Applied and Environmental Microbiology 69, 7453–7461.

Meroth, C.B., Walter, J., Hertel, C., Brandt, M.J., Hammes, W.P., 2003b. Monitoring the bacterial population dynamics in sourdough fermentation processes by using PCR-denaturing gradient gel electrophoresis. Applied and Environmental Microbiology 69, 475–482.

Metaxopoulos, J., Samelis, J., Papadelli, M., 2001. Technological and microbiological evaluation of traditional processes as modified for the industrial manufacturing of dry fermented sausage in Greece. Italian Journal of Food Science 13, 3–18.

Mills, D.A., Johannsen, E.A., Cocolin, L., 2002. Yeast diversity and persistence in botrytis-affected wine fermentation. Applied and Environmental Microbiology 68, 4884–4893.

Molly, K., Demeyer, D., Johansson, G., Raemaekers, M., Ghistelinek, M., Geenen, I., 1997. The importance of meat enzymes in ripening and flavour generation in dry fermented sausages. First results of a European project. Food Chemistry 59, 539–545.

Montel, M.C., Talon, R., Berdague', J.L., Cantonnet, M., 1993. Identification of *Staphylococcus* from French dry sausage. Letters in Applied Microbiology 15, 73–77.

Montel, M.C., Masson, F., Talon, R., 1998. Bacterial rolo in flavour development. Proceedings, 44th International Congress on Meat Science and Technology, Barcelona, vol. 1, pp. 224–233. IRTA/Eurocarne.

Morot-Bizot, S., Talon, R., Leroy-Setrin, S., 2003. Development of specific primers for a rapid and accurate identification of *Staphylococcus xylosus*, a species used in food fermentation. Journal of Microbiological Methods 55, 279–286.

Muyzer, G., 1999. DGGE/TGGE a method for identifying genes from natural ecosystems. Current Opinions in Microbiology 2, 317–322.

Muyzer, G., Smalla, K., 1998. Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. Antonie Van Leeuwenhoek 73, 127–141.

Muyzer, G., De Waal, E.D., Uitterlinden, A.G., 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Applied and Environmental Microbiology 59, 695–700.

Muyzer, G., Hottentrager, S., Teske, A., Wawer, C., 1995. Denaturing gradient gel electrophoresis of PCR-amplified 16S rDNA — a new molecular approach to analyse the genetic diversity of mixed microbial communities. In: Akkermans, A.D.L., van Elsas, J.D., de Bruijn, F.J. (Eds.), Molecular Microbial Ecology Manual. Kluwer Academic Publishers, Dordrecht, pp. 1–23.

Myers, R.M., Maniatis, T., Lerman, L.S., 1987. Detection and localization of single base change by denaturing gel electrophoresis. Methods in Enzymology 155, 501–527.

Nissen, H., Dainty, R., 1995. Comparison of the use of rRNA probes and conventional methods in identifying strains of *Lactobacillus sake* and *L. curvatus* isolated from meat. International Journal of Food Microbiology 25, 311–315.

Nour, M., 1998. 16S-23S and 23S-5S intergenic spacer regions of lactobacilli: nucleotide sequence, secondary structure and comparative analysis. Research in Microbiology 149, 433–448.

Papamanoli, E., Kotzekidou, P., Tzanetakis, N., Litopoulou-Tzanetaki, E., 2002. Characterization of Micrococcaceae isolated from dry fermented sausages. Food Microbiology 19, 441–449.

Papamanoli, E., Tzanetakis, N., Lipopoulou-Tzanetaki, E., Kotzekidou, P., 2003. Characterization of lactic acid bacteria isolated from a Greek dry-fermented sausage in respect of their technological and probiotic properties. Meat Science 65, 859–867.

Parente, E., Griego, S., Crudele, M.A., 2001. Phenotypic diversity of lactic acid bacteria isolated from fermented sausages produced in Basilicata (southern Italy). Journal of Applied Microbiology 90, 943–952.

Pereira, C.I., Barreto Crespo, M.T., San Romao, M.V., 2001. Evidence for proteolytic activity and biogenic amines production in *Lactobacillus curvatus* and *L. homohiochii*. International Journal of Food Microbiology 68, 211–216.

Powell, H.A., Gooding, C.M., Garreit, S.D., Lund, B.M., McKee, R.A., 1994. Proteinase inhibition of the detection of *Listeria monocytogenes* in milk using the polymerase chain reaction. Letters in Applied Microbiology 18, 59–61.

Randazzo, C.L., Torriani, S., Akkermans, A.D.L., de Vos, W.M., Vaughan, E.E., 2002. Diversity, dynamics, and activity of bacterial communities during production of an artisanal Sicilian cheese as evaluated by 16S rRNA analysis. Applied and Environmental Microbiology 68, 1882–1892.

Rantsiou, K., Comi, G., Cocolin, L., 2004. The *rpo*B gene as a target for PCR-DGGE analysis to follow lactic acid bacteria population dynamics during food fermentations. Food Microbiology 21, 481–487.

Rantsiou, K., Drosinos, E.H., Gialitaki, M., Urso, R., Krommer, J., Gasparik-Reichardt, J., Toth, S., Metaxopoulos, I., Comi, G., Cocolin, L., 2005a. Molecular characterization of *Lactobacillus* species isolated from naturally fermented sausages produced in Greece, Hungary and Italy. Food Microbiology 22, 19–28.

Rantsiou, K., Iacumin, L., Cantoni, C., Comi, G., Cocolin, L., 2005b. Ecology and characterization by molecular methods of *Staphylococcus* species isolated from fresh sausages. International Journal of Food Microbiology 97, 277–284.

Rantsiou, K., Urso, R., Iacumin, L., Cantoni, C., Cattaneo, P., Comi, G., Cocolin, L., 2005c. Culture dependent and independent methods to investigate the microbial ecology of Italian fermented sausages. Applied and Environmental Microbiology 71, 1977–1986.

Rebecchi, A., Crivori, S., Sarra, P.G., Cocconcelli, P.S., 1998. Physiological and molecular techniques for the study of bacterial community development in sausage fermentation. Journal of Applied Microbiology 84, 1043–1049.

Reuter, G., 1972. Experimental ripening of dry sausages using lactobacilli and micrococci starter cultures. Fleischwirtschaft 52, 465–468, 471–473.

Rodriguez, M., Nunez, F., Cordoba, J.J., Sanabria, C., Bermudez, E., Asensio, M.A., 1994. Characterization of *Staphylococcus* spp. and *Micrococcus* spp. isolated from Iberian ham throughout the ripening process. International Journal of Food Microbiology 24, 329–335.

Rossen, L., Norskov, P., Holmstrom, K., Rasmussen, O.F., 1992. Inhibition of PCR by components of food samples, microbial diagnostic assays and DNA-extraction solutions. International Journal of Food Microbiology 17, 37–45.

Rossi, F., Tofalo, R., Torriani, S., Suzzi, G., 2001. Identification by 16S-23S rDNA intergenic region amplification, genotypic and phenotypic clustering of *Staphylococcus xylosus* strains from dry sausages. Journal of Applied Microbiology 90, 365–371.

Samelis, J., Aggelis, G., Metaxopoulos, J., 1993. Lipolytic and microbial changes during the natural fermentation and ripening of Greek dry sausages. Meat Science 35, 371–385.

Samelis, J., Maurogenakis, F., Metaxopoulos, J., 1994. Characterisation of lactic acid bacteria isolated from naturally fermented Greek dry salami. International Journal of Food Microbiology 23, 179–196.

Samelis, J., Tsakalidou, E., Metaxopoulos, J., Kalantzopoulos, G., 1995. Differentiation of *Lactobacillus sake* and *Lact. curvatus* isolated from

naturally fermented Greek dry salami by SDS-PAGE of whole cell proteins. Journal of Applied Bacteriology 78, 157–163.

Samelis, J., Metaxopoulos, J., Vlassi, M., Pappa, A., 1998. Stability and safety of traditional Greek salami — a microbiological ecology study. International Journal of Food Microbiology 23, 179–196.

Santos, E.M., Gonzalez-Fernandez, C., Jaime, I., Rovira, J., 1998. Comparative study of lactic acid bacteria house flora isolated in different varieties of "chorizo". International Journal of Food Microbiology 39, 123–128.

Santos, N.N., Santos-Mendosa, R.C., Sanz, Y., Bolumar, T., Aristoy, M.-C., Toldrà, F., 2001. Hydrolysis of pork muscle sarcoplasmatic proteins by *Debaryomyces hansenii*. International Journal of Food Microbiology 68, 199–206.

Sanz, B., Selgas, D., Parejo, I., Ordonez, J.A., 1988. Characteristics of lactobacilli isolated from dry fermented sausages. International Journal of Food Microbiology 6, 199–205.

Sanz, Y., Hernandez, M., Ferrus, M.A., Hernandez, J., 1998. Characterization of *Lactobacillus sake* isolates from dry-cured sausage by restriction fragment length polymorphism analysis of the 16S rRNA gene. Journal of Applied Microbiology 84, 600–6006.

Sanz, Y., Fadda, S., Vignolo, G., Aristoy, M.-C., Oliver, G., Toldra', F., 1999. Hydrolysis of muscle myofibrillar proteins by *Lactobacillus curvatus* and *Lactobacillus sake*. International Journal of Food Microbiology 53, 115–125.

Schleifer, K.H., 1986. Gram positive cocci. In: Sneath, P.H.A., Mair, N.S., Holt, J.G. (Eds.), Bergey's Manual of Systematic Bacteriology, vol. 2. Williams & Wilkins, Baltimore, pp. 999–1003.

Talon, R., Walter, D., Chartier, S., Barriere, C., Montel, M.C., 1999. Effect of nitrate and incubation conditions on the production of catalase and nitrate reductase by staphylococci. International Journal of Food Microbiology 52, 47–56.

Torriani, S., Dellaglio, F., Palummeri, M., 1990. Characterization of lactobacilli isolated from Italian sausages. Annali di Microbiologia 40, 225–233.

van Beek, S., Priest, F.G., 2002. Evolution of the lactic acid bacterial community during malt whisky fermentation: a polyphasic study. Applied and Environmental Microbiology 68, 297–305.

van der Gucht, K., Sabbe, K., de Meester, L., Vloemens, N., Zwart, G., Gillis, M., Vyverman, W., 2001. Contrasting bacterioplankton community composition and seasonal dynamics in two neighboring hypertrophic freshwater lakes. Environmental Microbiology 3, 680–690.

Woese, C.R., 1987. Bacterial evolution. Microbiology Reviews 51, 221–271.

Yost, C.K., Nattress, F.M., 2000. The use of multiplex PCR reactions to characterize populations of lactic acid bacteria associated with meat spoilage. Letters in Applied Microbiology 31, 129–133.

Yu, Z., Morrison, M., 2004. Comparison of different hypervariable regions of *rrs* genes for use in fingerprinting of microbial communities by PCR-denaturing gradient gel electrophoresis. Applied and Environmental Microbiology 70, 4800–4806.

Zhong, W., Millsap, K., Bialkowska-Hobrzanska, H., Reid, G., 1998. Differentiation of *Lactobacillus* species by molecular typing. Applied and Environmental Microbiology 64, 2418–2423.