Chapter 9

Application of Molecular Methods in Fermented Meat Microbiota: Biotechnological and Food Safety Benefits

Maria Grazia Bonomo*, Caterina Cafaro, Giovanni Salzano

1 INTRODUCTION

There is a great variety and diversity of fermented meat products all over the world, as a consequence of different formulations used in their production. The ingredients together with manufacturing practices and fermentation techniques are the main factors that lead to products with specific organoleptic profiles and physico-chemical characteristics, making them unique (Bonomo et al. 2008, Cocolin et al. 2008).

Nowadays, fermented meat product manufacture is a very important part of the meat industry in many countries and the use of starter cultures was become common in the manufacture of several types of fermented products. However, many typical fermented products are still produced with artisanal technologies without selected starters (Fonseca et al. 2013). Artisanal products have greater quality than those from controlled fermentations inoculated with industrial starters and possess

*For Correspondence: Dipartimento di Scienze, Università degli Studi della Basilicata, Viale dell'Ateneo Lucano 10, 85100 Potenza, Italy, Email: mariagrazia.bonomo@unibas.it

distinctive qualities, due especially to the specific composition of the indigenous microbiota (Lebert et al. 2007, Martin et al. 2007, Talon et al. 2008). Therefore, commercial starter cultures are not always able to compete well with this house flora so that their use often results in loss of desirable sensory properties. So, appropriate cultures have to be selected from indigenous microorganisms, more competitive, well adapted to the particular product and to the specific production technology, and with high metabolic capacities which can beneficially affect product quality and safety, preserving their typicity (Leroy et al. 2006, Bonomo et al. 2009, Babić et al. 2011).

In the last decade, the study of the ecology of fermented meat products was of primary importance to understand the physical and chemical changes occurring during fermentation and ripening (Comi et al. 2005, Lücke 1985). In recent years, an increasing number of studies was focused on the isolation and identification of autochthonous functional starter cultures, with the aim of developing new functional meat products, including microorganisms that generate aroma compounds, health-promoting compounds, bacteriocins and other antimicrobials, possess probiotic qualities, or lack negative properties such as the production of biogenic amines and toxic compounds (Leroy et al. 2006, Ammor and Mayo 2007, Bernardeau et al. 2006, Noonpakdee et al. 2003, Bernardeau et al. 2008, Babić et al. 2011).

Meat fermented products are the result of a complex microbiological activity that mainly consists of a lactic fermentation and several characteristic biochemical changes in which the indispensable participants are lactic acid bacteria (LAB) and coagulase-negative cocci (CoNS) (Rantsiou and Cocolin 2008, Palavecino Prpich et al. 2015). The LAB play an important technological role in meat preservation and fermentation; they decrease the pH by lactic acid production, produce bacteriocins to suppress the growth of spoilage or pathogenic microorganisms, provide diversity of sensory properties by modification of raw material, contribute to the development of flavour, colour and texture and improve the safety, stability and shelf life of meat products (Fontana et al. 2005a, Frece et al. 2005, Frece et al. 2009, Hugas and Monfort 1997, Lücke 2000). Coagulase-negative cocci contribute to the development of sensory properties (flavour, texture and colour) and affect the quality and the stability of the products by their biochemical-metabolic properties, such as nitrate reduction, proteolytic and lipolytic activity (Mauriello et al. 2004, Olesen et al. 2004, Tjener et al. 2004). The ability of CoNS to produce antimicrobial compounds may improve the safety and shelf-life of sausages (Martín et al. 2007, Simonova et al. 2006, Leroy et al. 2009, 2010).

In the last 20 years, the advancements in molecular biology have revolutionized the study of microbial biodiversity with a new range of

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techniques 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 quantification of microorganisms (Cocolin et al. 2011, Cocolin et al. 2008).

Nowadays there is a growing interest in developing novel foods containing probiotic microorganisms, such as bifidobacteria and LAB. Such functional cultures may offer organoleptic, technological and nutritional advantages, but more importantly confer a health benefit on the host, as the probiotics administration has been linked to the treatment of various diseases (Deshpandea et al. 2011). The mechanisms and the efficacy of a probiotic effect often depend on the interactions with the host microbiota or with the immuno-competent cells of the intestinal mucosa (Saad et al. 2013).

A properly designed strategy for the incorporation of probiotic microorganisms into foods (formulation strategies, processing, stability and organoleptic quality issues) is a key factor in the development of functional products. Although encapsulation systems have largely been exploited in the pharmaceutical (e.g. drug and vaccine delivery) and agricultural sector (e.g. fertilizers), the food industry has only recently become aware of the immense benefits that these technologies are able to offer (Champagne et al. 2010). Insertion of beneficial bacteria into a food matrix presents a new challenge, not only because of their interactions with other constituents, but also because of the severe conditions often employed during food processing and storage, which might lead to important losses in viability, as probiotic strains are very often thermally labile (on heating and/or freezing) and sensitive to acidity, oxygen or other food constituents (e.g. salts).

There were only a few attempts at incorporating probiotic cultures in dry-fermented sausages (De Vuyst et al. 2008, Muthukumarasamy and Holley 2006) and the results are still considered preliminary for evaluating the effect of probiotic fermented meats on human health. Most concerns are associated with the survival of the probiotic strains during the manufacturing process and detection in high numbers in the end-product (De Vuyst et al. 2008, Leroy et al. 2006). The major difficulty is the monitoring of the probiotic cultures survival in foods, due to the lack of accurate, reliable, convenient and sensitive methods of identification to distinguish the strains of interest among other closely related microorganisms present in the products.

This chapter will discuss the most recent and advanced molecular methods applied to identify, characterize and profile microbial diversity and dynamics of fermented meat products. Understanding the microbial biodiversity and ecology can allow a better control of the transformation

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process, resulting in products with high quality and safety and unique sensory characteristics and facilitating the development of autochthonous starters.

2 MOLECULAR APPROACHES IN FERMENTED MEAT MICROBIOTA

Molecular techniques have been used as an effective method to identify and characterize the microbiota of fermented meat products for the last 20 years. Developments in the field of molecular biology, allowed for new methods to become available, which could be applied to better understand dynamics and diversity of the microorganisms in fermented meat products. Today it is possible to detect, identify and quantify microorganisms by targeting their nucleic acids (Cocolin et al. 2011).

Nucleic acids can be analyzed either from isolates obtained from the food matrix by traditional microbiological methods (culture-dependent techniques), or by direct extraction from the food sample (culture-independent techniques). In the last 10 years, several studies have underlined that, often, there are significant differences between the results obtained with culture-independent and -dependent methods (Cocolin et al. 2004, Cocolin et al. 2011, Doyle and Buchanan 2013, Ceuppens et al. 2014).

There is a scientific consensus on the fact that culture-dependent methods are not able to properly describe the diversity of complex ecosystems; populations that are present in low numbers or that are in a stressed or injured state will most probably be unintentionally excluded from consideration if traditional microbiological methods are used. Moreover, the cells in a viable but not culturable (VBNC) state cannot be detected, because of their incapability to form colonies on microbiological media (Cocolin et al. 2008, Cocolin et al. 2004). However, also culture-independent methods possess some limitations due to generally high limits of detection, thereby minor populations are not taken into consideration.

Culture-independent approaches are employed to study the ecology and biodiversity in food fermentations, using DNA and RNA as target molecules, that have different properties and meaning and allow obtaining different kind of results. Studying the DNA of a microbial ecosystem will allow definition of the microbial ecology and diversity as DNA is a very stable molecule and it is long present also after the cell has died, while the RNA analysis will highlight more properly the microbial populations that are metabolically active, thereby contributing to the fermentation process as RNA, and especially messenger RNA (mRNA), can have very short life (Cocolin et al. 2008, Chakraborty et al. 2014).

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Recently the combination of culture-dependent and culture-independent molecular methods have become the preferred approach for determining and analyzing the species composition of different microbial communities (Ercolini et al. 2001a, b, Temmerman et al. 2003, 2004, Silvestri et al. 2007, Kesmen et al. 2012).

2.1 Culture-Independent Methods

The advent of culture-independent methods to characterize the microbiota from fermented food products allow to analyze multiple samples simultaneously without the need for preceding culturing or for prior knowledge of the ecosystems diversity (Cocolin et al. 2001). In this way the investigation of microbial ecology has dramatically changed and this process is in constant evolution (Solieri et al. 2013). For a long period, food-associated microorganisms and their dynamics have been studied through culture based-methods (Doyle and Buchanan 2013). However, these revealed to be often weak to accomplish a complete microbial characterization of many ecosystems, among which foodstuffs (Ceuppens et al. 2014). Problems and shortcomings of culturing methods basically involve the underestimation of microbial diversity, and even the failure of a precise detection of some species or genera. The introduction of molecular technologies permitted to identify food related microorganisms and to evaluate their relative abundance, providing a fast, accurate and economic detection tool increasingly important in food microbiology, complementing or substituting classical methods (Ceuppens et al. 2014, Chakraborty et al. 2014, Galimberti et al. 2015).

2.1.1 Denaturing Gradient Gel Electrophoresis (DGGE)

The culture-independent method that has become more popular to study the diversity of microbial communities and has been more extensively applied to sausage fermentation is the analysis of PCR products by using denaturing gradient gel electrophoresis (DGGE) (Rantsiou and Cocolin 2008, Cocolin et al. 2001).

The technique consists of the electrophoretic separation of PCR-generated double stranded DNA in a polyacrylamide gel containing a gradient of chemical denaturants (urea and formamide); when the DNA molecule meets with an appropriate denaturant concentration, a sequence-dependent, partial denaturation of the double strand occurs and this conformation change of the DNA structure causes a reduced migration rate of the molecule. When the method is used for microbial profiling, PCR is carried out with universal primers, able to prime amplification for all the microbes present in the sample, and then, the complex mixture of the DNA molecules obtained can be differentiated and characterized by separation on denaturing gradient gels.

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Every single band that is visible in DGGE gels represents a component of the microbiota; the more bands are visible, the more complex is the ecosystem. By using this method, it is possible not only to profile the microbial populations, but also to follow their dynamics during time. Modern image analysis systems have proven to be of value for the analysis of DGGE bands and their associated patterns. It should be noted that these methods are not quantitative (Rantsiou and Cocolin 2006). Direct PCR amplification of different regions of the 16S rRNA gene and subsequent analysis by DGGE has been used to study the ecology of the microbial processes involved in the production of many Italian fermented sausages (Aquilanti et al. 2007, Cocolin et al. 2009, Cocolin et al. 2001, Rantsiou et al. 2005, Silvestri et al. 2007, Villani et al. 2007), but studies on the fermentation dynamics of Argentinean (Fontana et al. 2005a, Fontana et al. 2005b) and Portuguese (Albano et al. 2008), Greek (Sidira et al. 2014), Vietnamese (Nguyen et al. 2013) and Turkish (Kesmen et al. 2012) sausages are available as well. Moreover, the use of DGGE in food microbiology was reviewed by Ercolini (2004) and recently by other authors (Cocolin et al. 2011, Galimberti et al. 2015) that checked the contribution of molecular methods for a better comprehension of complex food ecosystems as biodiversity and dynamics of meat fermentations.

It was reported that PCR-DGGE was the most suitable technique to investigate the microbial community in different food matrices in which the cultivation of many microorganisms is difficult or is thought to be impossible. However the detection limit of DGGE depends on the target species, the concentration of the other members of the microbial community and nature of the food matrix (Fontana et al. 2005b). Moreover the efficiency of DNA extraction and possible competition among templates during PCR amplification also affect the limit of detection (Ercolini 2004).

The study of Cocolin et al. (2001) was the first work that used the direct DGGE analysis to monitor the bacterial population dynamics during natural fermentation of Italian sausages. The PCR-amplified V1 region of the 16S rRNA gene analysis highlighted how fermentation of sausages is a highly competitive process in which a wide species diversity can be found at the beginning of fermentation and then a rapid evolution of the predominant populations is observed evidencing the presence of the active population responsible for the changes that occurred during ripening of the sausages. This was confirmed also by other studies, for example the general consideration that resulted from the study of Rantsiou et al. (2005) was that the main differences detected in the ecology, between traditional fermented sausages from the same region of Italy but three different plants, were not represented by the microbial species identified by band sequencing, but by their relative

distribution between fermentations. In general, in different studies, targeting the V1 and V3 regions 16S rRNA, it was possible to identify the main lactic acid bacteria and staphylococci species and to prove the important role that these microbial groups have in the specific fermented sausages production (Fontana et al. 2005b, Villani et al. 2007, Albano et al. 2008, Cocolin et al. 2009). DGGE was used in monitoring the growth kinetics during the ripening process and the effects of these on the safety and quality, allowing tracking of the microbial 'typicity' and collecting of important information for the designing of autochthonous starter cultures (Aquilanti et al. 2007, Albano et al. 2008).

Recently, novel fermented meat products were investigated, always, preserving the combination of culture-dependent and -independent molecular methods to characterize the typical microbiota attributed to the specific conditions prevalent at the location of production for a better understanding of their influence on the typical characteristics of the product (Nguyen et al. 2013, Kesmen et al. 2012). As a result of these studies, it was concluded that polyphasic strategies are necessary for accurate and reliable screening of the microbial composition of fermented products. This application could provide an opportunity to better understand and control the transformation process during fermentation. Moreover the profiling of bacterial populations occurring in these artisanal products can be useful to determine the technologically important strains to be employed as an autochthonous starter culture to obtain high quality and safety properties and the desired sensory profiles in the final product (Nguyen et al. 2013, Kesmen et al. 2012).

Moreover, recently, fermentation has been shown to have not only preservative effects and the ability of aiding the modification of the physicochemical properties of different foods but also the capability to provide significant impact on the nutritional quality and functional performances of the raw material (Kos et al. 2003), so it has been observed a growing interest in developing novel fermented meat products containing probiotic microorganisms, with functional properties that can offer organoleptic, technological and nutritional advantages and particularly confer a health benefit on the host. A new frontier goal for fermented meat is the use of functional starter cultures, i.e. starter cultures able to improve food safety, and to preserve the typical sensory quality of traditional sausages but with an "added function", potential health benefits, as proposed by some authors (Babić et al. 2011, Bevilacqua et al. 2015).

A recent study of Sidira et al. (2014) proved a novel and interesting picture of fermented meat products in which the use of immobilized *Lactobacillus casei* on wheat as probiotic starter culture in dry-fermented sausages was assessed. Insertion of beneficial bacteria into a food matrix presents a new challenge, not only because of their interactions with

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other constituents, but also because of the severe conditions often employed during food processing and storage, which might lead to important losses in viability; therefore, to overcome such deficiencies, immobilization techniques are usually applied in order to maintain cell viability, activity and functionality, in order to allow the formulation of new types of foods fortified with immobilized health-promoting bacteria that are only released upon reaching the human gut (Champagne et al. 2010, Bosnea et al. 2009, Charalampopoulos et al. 2003).

A few attempts at incorporating probiotic cultures in dry-fermented sausages were performed (De Vuyst et al. 2008, Muthukumarasamy and Holley 2006), and the results are still considered preliminary for evaluating the effect of probiotic fermented meats on human health; moreover, the monitoring of the probiotic cultures survival in foods is hampered by the lack of accurate, reliable, convenient and sensitive methods of identification to distinguish the strains of interest among other closely related microorganisms present in the products. For this reason, Sidira et al. (2014) investigated the microbial interactions and dynamics in a novel fermented meat product by V1 region of the 16S rRNA gene DGGE analysis during manufacture and ripening. This analysis allowed documenting the survival of *Lb. casei* cells at the required levels for providing the health benefits at the time of consumption (during ripening and after mild heat treatment), repression of spoilage and pathogenic bacteria, improvement of quality characteristics and extension of products' shelf-life.

The application of PCR–DGGE in the field of fermented sausages offers a better understanding of the biodiversity and dynamics of the populations involved in the transformation. However it should be mentioned that pitfalls, associated with sampling, DNA extraction, DNA purity, PCR conditions, formation of heteroduplex and chimeric molecules, may still exist, thereby the results obtained need to be verified and validated (Ercolini 2004). One important aspect that has to be taken into consideration when applying DGGE in food fermentation is the sensitivity limit. It has been demonstrated that populations that are below 10^3–10^4 colony forming units (cfu)/g will not be detected (Cocolin et al. 2001). This is especially valid when in the same ecosystem two populations, one at high and the other at low counts, exist, as it usually happens in sausage fermentations. Moreover, due to the extensive application of sequencing, databases have seen tremendous growth of the sequences deposited. Often, these entries are classified as 'unculturable microorganism', since they have been detected only by culture-independent methods and no significant similarity to available sequences was obtained. This aspect introduces potential difficulties in the understanding of the ecology of fermented foods and at the same time underlines the need to improve the traditional cultivation methods (Cocolin et al. 2011).

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2.1.2 Next Generation Sequencing (NGS)

Since advances in technology have always driven discoveries and changes in microorganism taxonomy, taxonomic identification is an issue of primary importance when approaching the study of food microbiota. In this scenario, genomics now underlies a renaissance in food microbiology therefore accelerating food safety monitoring and food production processes (Ceuppens et al. 2014). Microbial taxonomy directly influences a number of basic scientific and applied fields where microorganisms are involved (Tautz et al. 2003) including food production, conservation and probiotic activity. Depending on the level of investigation required, the taxonomic resolution of microorganisms can vary. Aiming to differentiate microorganisms at the species level, methods based on DNA sequencing are currently the most adopted. In many cases, when a fast and accurate response is needed, a 'DNA barcoding-like' approach is the most reliable (Chakraborty et al. 2014). Many scientists used 16S rRNA gene as a universal marker for species-level typing of microorganisms (Bokulich et al. 2012, Claesson et al. 2010, Janda and Abbot 2007). This genomic region is considered a 'bacterial barcode' due to its peculiar properties (Patel 2001): it is present in all the bacterial species, it contains sufficient information (1500 bp long) to differentiate species and, in some cases, strains (Muñoz-Quezada et al. 2013) and finally, the 16S rRNA relies upon an impressive archive of reference sequences such as Greengenes (De Santis et al. 2006) and SILVA (Pruesse et al. 2007). Amplicons belonging to whole genomic extraction conducted on food products matrices are sequenced and the reads are compared to reference databases to identify the Operational Taxonomic Units (OTUs).

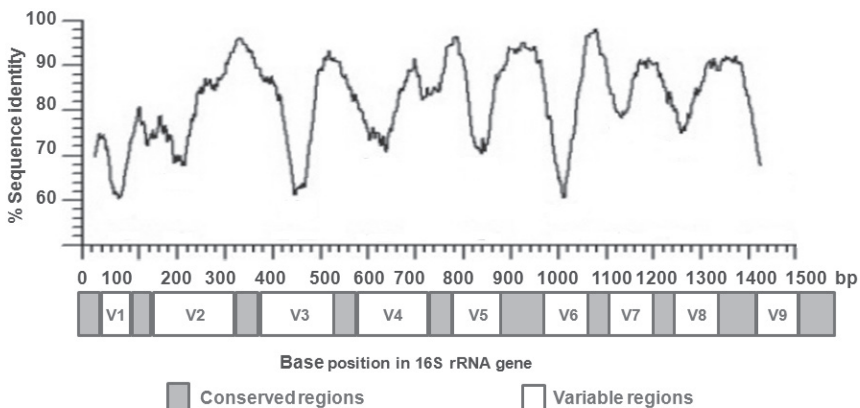

Figure 1 Schematic overview of the 16S rRNA gene. The Sequence identity of the 16S rRNA gene of more than 6,000 bacteria is shown. V1–V9 are the hypervariable regions while the others are the conserved regions (modified from Wahl et al. 2015).

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Figure 1 shows the nine hypervariable regions (V1–V9) contained in the approximately 1500 bp long 16S rRNA which can be used for identification of bacteria; a part of the gene is amplified by PCR with primers binding in highly conserved regions, subsequently, the resulting PCR product spanning one or more hypervariable regions is sequenced and used for taxonomic classification.

Progresses in sequencing technologies and bioinformatics analysis of data, led nowadays to a more complex scenario of food microbial communities, offering a panel of analytical tools able to screen the whole microbial community of food matrices. The use of universal markers produces several DNA barcode fragments, corresponding to the each bacterial species present in a food sample. With the ultimate goal of characterizing the complete spectrum of microorganisms, several novel approaches, referred to as 'Next Generation Sequencing' (NGS) and, more recently, 'High Throughput Sequencing' (HTS), have been developed (Ercolini 2013, Mayo et al. 2014, Solieri et al. 2013, Galimberti et al. 2015).

Food microbiology deals with the study of microorganisms that have both beneficial and deleterious effects on the quality and safety of food products. The fast and low-cost NGS approaches have revolutionized microbial taxonomy and classification and have changed the landscape of genome sequencing projects for food-associated microbial species (Coenye et al. 2005). The NGS-driven advances have been exploited mainly to re-sequence strains and individuals for which reference genome sequences are available in order to sample genomic diversity within microbial species. The NGS approaches have greatly increased the ability of researchers to profile food microbial communities, as well as to elucidate the molecular mechanisms of interesting functionalities in food ecosystems. These applications enable the culture-independent sequencing of collective sets of DNA or RNA molecules obtained from mixed microbial communities to determine their content (Solieri et al. 2013). Figure 2 presents a general flow chart of Next-Generation Sequencing (NGS) applications in food microbiology.

NGS techniques have promoted the emergence of new, high-throughput technologies, such as genomics, metagenomics, transcriptomics and metatranscriptomics, etc. As compared to previous culture-independent methods, the number of nucleic acid sequences analyzed by NGS techniques is exceedingly higher, allowing a deeper description of the microbial constituents of the ecosystems. These technologies can be used in two substantially different ways: sequencing the total microbial nucleic acids (shotgun sequencing) and gene-specific sequencing (targeted sequencing). For the latter, segments of highly conserved DNA or cDNA sequences are first amplified by PCR using universal or group-specific primers. Targeted techniques provide a

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snapshot of the diversity and phylogeny of the different elements making up microbial populations. The term phylobiome has been introduced recently to refer to the phylogenetic information gathered using this approach (van Hijum et al. 2013). In addition, shotgun techniques inform on the genetics and functional capabilities of the microbial constituents of food ecosystems, providing insights into the number and potential function of genes within the community (Wilmes et al. 2009, Solieri et al. 2013). Both shotgun and targeted techniques have already been used to study the microbiology of a series of foods and food fermentations, and pertinent reviews have recently been compiled (Solieri et al. 2013, Liu 2011, Bokulich and Mills 2012, Ercolini 2013). However, research in this area is so active that findings must be continually reviewed, and the current and potential applications of these constantly updated.

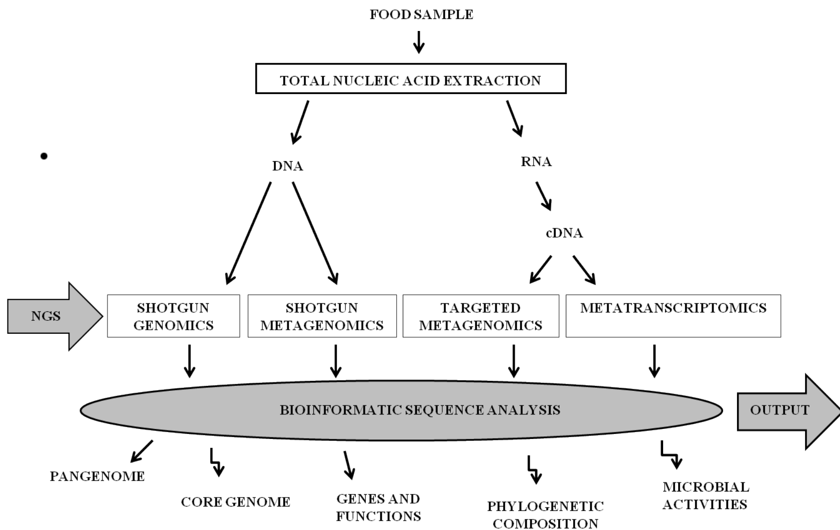

Figure 2 General flow chart of Next-Generation Sequencing (NGS) applications in food microbiology.

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Interesting review articles on various aspects of the impact of NGS technologies on food microbial genomics were drafted, and provided complete information about the most common NGS systems and platforms and then addressed how NGS techniques have been employed in the study of food microbiota and food fermentations, discussing their limits and perspectives. The most important findings are reviewed, including those made in the study of the microbiota of milk, fermented dairy products, and plant-, meat- and fish derived fermented foods (Ercolini 2013, Solieri et al. 2013, Mayo et al. 2014).

NGS platforms involve many different technologies (Glenn 2011) all of which generate large, genome-scale datasets. However, they differ

substantially in terms of their engineering, sequencing chemistry, output (length of reads, number of sequences), accuracy and cost. Current commercial platforms include the 454 (Roche), Illumina (Illumina), SOLiD and Ion Torrent (Life Technologies), and PacBio (Pacific Biosciences) systems. Comparisons of their advantages and disadvantages have recently been published (Liu et al. 2012, Quail et al. 2012). Many other 'third-generation' techniques, such as DNA nanoball sequencing, heliscope single molecule sequencing, nanopore DNA sequencing, tunneling current DNA sequencing, sequencing with mass spectrometry, and microscopy-based techniques, are currently under development (Schadt et al. 2010, Mayo et al. 2014, Galimberti et al. 2015).

In general, all of the NGS analysis platforms require three principal steps, each of which must be optimized, that are library preparation, template amplification and sequencing, and are shown in Figure 3 and described in details in Loman et al. (2012) and in Mayo et al. (2014).

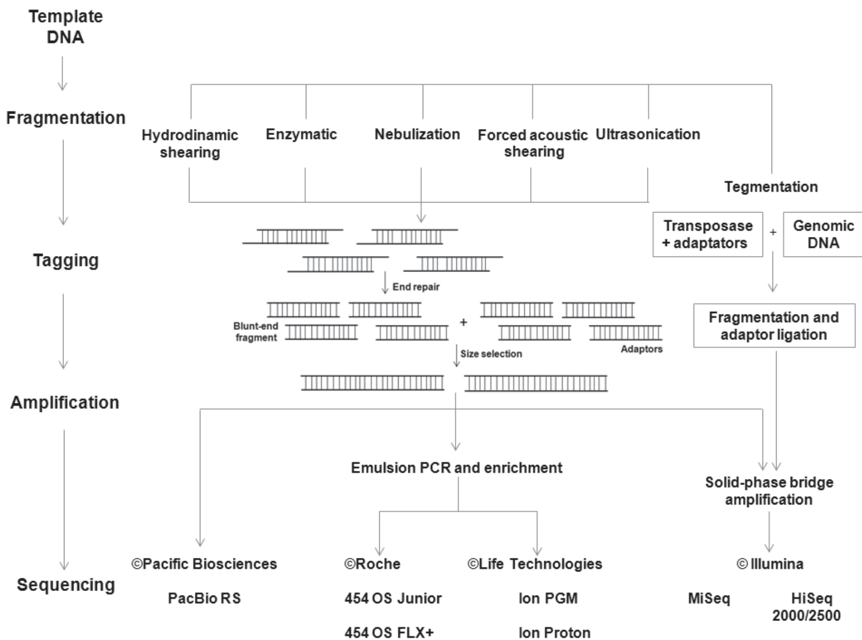

Figure 3 Next-Generation Sequencing (NGS) analysis platforms (modified from Loman et al. 2012).

Recently, various studies used NGS approaches to study the microbial ecosystem (in terms of diversity and dynamics) of different fermented foods (Ercolini et al. 2012, Masoud et al. 2011, Bokulich and Mills 2012, Bokulich et al. 2012, Jung et al. 2012, Kiyohara et al. 2011, Koyanagi et al. 2011, Roh et al. 2010, Nam et al. 2012a, Nam et al. 2012b, Polka

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et al. 2015), and, in most cases, the obtained results could be of great impact on the food supply chain to improve industrial biotransformation processes, enhance quality of final products, extend the shelf-life and valuating local productions.

Currently, some reports on meat and meat products have been published; their basic goal was to characterize microbial diversity and community dynamics in order to understand the relationships between microorganisms and their impact on food sensorial properties and safety. The application of NGS approach can contribute to describe microorganisms and their activities so as to work out their roles in changes in the quality of fresh foods. Some studies revealed an high bacterial diversity in beef, minced and other meat sort, showing how microbial diversity, growth dynamics, and metabolite production can change according to different storage packaging conditions and how the association between microbial development and chemical changes occurring during the storage of meat is a potential means of revealing indicators of meat quality or freshness (De Filippis et al. 2013, Ercolini et al. 2011, Stoops et al. 2015, Kiermeier et al. 2013, Xiao et al. 2013).

Similarly, Nieminen et al. (2012) compared microbial communities in marinated and unmarinated broiler meat by metagenomic analysis, evidencing a number of bacterial taxa that have not been associated with late shelf-life meat before. In this study, the sequencing depth allowed observation of functional differences between metagenomes that reflect the differences in phylogenetic composition of the bacterial communities rather than functions that could be specifically related to bacterial growth in the investigated meat preparations. A more informative comparison of spoilage-related functions in different meat products could be achieved by studying the actively expressed genes using metatranscriptomics.

In the study of Chaillou et al. (2014), bacterial communities associated to spoilage of four meat products were explored by V1–V3 region of the 16S rRNA gene pyrosequencing to describe microbial diversity at the species level as spoilage could be species-dependent. They recognized reservoirs of spoilage bacteria and deduced the impact of different storage conditions and food properties on these communities, with novel species that could be involved in spoilage and unpredicted assemblages of dominant species.

Investigation of the bacterial communities involved in fermented meat products can be carried out by microbiological and molecular methods. The latter approaches are now being revolutionized by the introduction of NGS, most specifically Illumina platforms which provide million of reads up to 300 × 2 bp length, with further advancements expected in the near future (Bokulich and Mills 2012, Shokralla et al. 2012). We are rapidly moving to the postgenomic era, when a complete assessment of the genes present in a certain sample will be obtained,

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and their expression and activity assessed by metatrascriptomic and metaproteomics. An important step in this direction is represented by the quantitative assessment of bacterial taxa, which can be achieved by sequencing of 16S rRNA amplicons: the identification on the basis of 16S NGS data of the species present in a food sample can greatly support metagenomic analyses to be conducted on the whole microbial DNA. In the work of Polka et al. (2015), Illumina MiSeq approach was applied to characterize the bacterial community of Salame Piacentino PDO (Protected Designation of Origin), a fermented sausage from different factories and at different ripening stages.

Culture-based microbiological analyses and PCR-DGGE were carried out in order to be compared with NGS results obtained by Illumina MiSeq analyses of the two bacterial 16S hypervariable regions V3 and V4. The initial approach was the same as PCR-DGGE, i.e., the amplification of hypervariable regions in a gene marker of interest such as the 16S rRNA, but rather than being separated on denaturing gel and eventually sequenced after cloning or cutting of bands of interests, amplicons were directly analyzed on an Illumina MySeq platform employing a 250 × 2 bp paired reads chemistry. As, previously (Vasileiadis et al. 2012), it has been demonstrated that analyses of single hypervariable regions can support a good taxonomical assessment of bacterial diversity, Polka et al. (2015) showed that a manual curation of 16S rRNA databases such as the GreenGenes database can greatly improve the taxonomical assignment of sequences covering the V3 and V4 regions of bacteria; a manual amendment of the database by adding reference sequences of genera that were found in salami samples, lead to a taxonomical assignment up to the species level for 99.4% of the 722.196 bacterial sequences retrieved from the salami. These results proved that the microbial community composition of dry-fermented sausages is largely composed of known species whose 16S sequences are already available in public databases; at methodological level, it also demonstrated that manual curation of reference database is very important to improve the taxonomical classification of NGS data, and that the sequencing of two hypervariable 16S regions permitted by Illumina technology is sufficient for diversity assessment of these fermented foods. The comparison between NGS and PCR-DGGE approaches showed that NGS confirms, with a better resolution and quantitative assessments, the samples patterns identified by PCR-DGGE. The greater performance of NGS was fully appreciated when taxonomical analyses were carried out; NGS also allowed identifying a diversity of species that is not appreciated by PCR-DGGE. PCR-DGGE, in fact, did not allow reliable quantification of population abundances, and the sequencing of excised bands identified only the main species, while the whole data set obtained by NGS analyses presented 13 main families and 98 rare ones, 27 of

which were present in at least 10% of the samples, even if, most probably, many of these sequences belong to environmental contaminants that do not play an ecological role in the sausage fermentation and with casings that were confirmed to be the major sources of rare species; since in local DOP products the use of casings from pig intestine is mandatory, this result confirm the importance of such practice to guarantee and preserve the bacterial diversity in fermented raw meats (Polka et al. 2015). This highlights the great potential of the NGS application to microbial ecology of fermented meat products to gain a complete and in-depth picture of the bacterial species and identify species that cannot be detected with classical microbiological and molecular methods.

Moreover, RNA-based analysis can significantly increase the ability to identify the impact of the microbial population on organoleptic characteristics of typical food products; when RNA is analyzed, the microbial populations that are metabolically active can be potentially detected and identified, and these are the populations that contribute the most to the fermentation process (Cocolin et al. 2013). In a recent study (Greppi et al. 2015) the diversity of metabolically active microbiota occurring during the natural fermentation of a traditional Piedmontese sausage was evaluated by using RT-PCR-DGGE coupled with RNA-based pyrosequencing of 16S rRNA gene. As to the study of Połka et al. (2015) on typical Italian dry-fermented sausages, that reported the presence of 32 different *Staphylococcaceae* and 33 *Lactobacillaceae* identified by using DNA-based NGS with DGGE techniques, Greppi et al. (2015) showed that only a few species belonging to those genera may be metabolically active and can really contribute to determine the final characteristics of the products.

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2.2 Culture-Dependent Methods

Since the nineties, molecular techniques for the identification and characterization of microorganisms isolated from fermented meat products, started to be used side by side or to substitute morphological, phenotypical and biochemical tests. Although the different approaches, in most of the cases, arrived at similar results, molecular techniques immediately showed a higher level of reproducibility, automatism and rapidity (Cocolin et al. 2008, 2011).

There is an important distinction between culture-dependent and -independent methods, as already mentioned; the culture-dependent methods are commonly used to molecularly identify and characterize microbial isolates, while the culture-independent ones are used to profile directly the microbial populations, dynamics and changes in the microbial ecology during fermentation and ripening processes (Cocolin et al. 2008). In recent decades, the study of fermented meat products

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microflora was carried out by different studies, most of which reported the molecular identification and characterization of isolated LAB and CNC strains considered the technologically relevant microbial groups in the fermented meat products (Ben Amor et al. 2007, Olive and Bean 1999, Temmerman et al. 2004, Kesmen et al. 2012). The 16S rRNA is the gene more often targeted for the identification of isolates, as it is common to all bacteria, it possesses variable and conserved regions used for the differentiation, it is as an evolutionary clock that provides phylogenetic information and, also, a large database of sequences is already available (Singh et al. 2009).

The increasing availability of the sequences of the 16S rRNA gene and the intergenic region between 16S rRNA and 23S rRNA genes allowed the development of different methods for the identification of microbial species of interest in the field of sausage fermentation (Rantsiou and Cocolin, 2008). Ribosomal RNA probes (Nissen and Dainty 1995, Hertel et al. 1991), species-specific PCR (Berthier and Ehrlich 1998, Yost and Nattress 2000, Blaiotta et al. 2003b, Corbiere Morot-Bizot et al. 2003, Rossi et al. 2001), randomly amplified polymorphic DNA (RAPD)-PCR (Berthier and Ehrlich 1999, Andrighetto et al. 2001, Comi et al. 2005), restriction analysis of amplified 16S rDNA gene (ARDRA)-PCR (Aymerich et al. 2006, Rantsiou and Cocolin 2006, Bonomo et al. 2008), restriction fragment length polymorphism (RFLP) analysis of the 16S rDNA gene (Sanz et al. 1998, Lee et al. 2004, Belgacem et al. 2009), multiplex PCR (Corbiere Morot-Bizot et al. 2004, Fonseca et al. 2013), PCR amplification of repetitive bacterial DNA elements (rep-PCR) (Gevers et al. 2001, Danilović et al. 2011), pulsed field gel electrophoresis (PFGE) (Di Maria et al. 2002) and DGGE (Cocolin et al. 2001b, Ercolini et al. 2001, Blaiotta et al. 2003a) have been applied for the identification and characterization of LAB and CNC isolated from fermented sausages.

The application of these molecular strategies has become a routine step for identification and characterization purposes in the last 20 years and a considerable literature, reporting in detail a review of the molecular methods applied in different studies, is offered (Rantsiou and Cocolin, 2008, Cocolin et al. 2008, Cocolin et al. 2011). All studies presented the predominance of *Lb. sakei*, *Lb. curvatus* and *Lb. plantarum,* considered the dominant flora responsible for the transformation process, as well as staphylococci such as *Staphylococcus xylosus*, *St. saprophyticus*, *St. carnosus* and *St. equorum* (Ammor et al. 2005, Aymerich et al. 2003, Cocolin et al. 2001, Comi et al. 2005, Papamanoli et al. 2003, Rantsiou et al. 2004, Rantsiou et al. 2005, Aymerich et al. 2003, Hugas et al. 1993, Papamanoli et al. 2003, Rebecchi et al. 1998, García Fontán et al. 2007a,b, Mauriello et al. 2004, Talon et al. 2007).

Some examples of the most recent analyses of microbiota in fermented meat products are discussed below. All of them highlighted

as, recently, the combination of culture-dependent and culture-independent molecular methods have become the preferred approach for determining and analyzing the species composition of targeted microbial communities (Kesmen et al. 2012, Fonseca et al. 2013, Nguyen et al. 2013, Federici et al. 2014, Wanangkarn et al. 2014, Kesmen et al. 2014). In all, the applied techniques are those established in the years for the identification and characterization but the novelty is that the analyzed microbiota was isolated from the not yet investigated products and also the new application purpose.

Kesmen et al. (2012) used different molecular strategies, based on both culture-dependent and culture-independent methods, to determine the total microbial diversity of the sucuk from different regions of Turkey by PCR-DGGE analysis of the PCR amplified V1 and V3 region of the 16S rRNA gene (rDNA), to identify and characterize LAB strains isolated from the sucuk samples and cultivated on opportune culture media by rep-PCR fingerprinting technique and then by the sequencing of the 16S rDNA and 16S–23S rRNA intergenic spacer regions. Similarly, the study of Nguyen et al. (2013) gave a more extensive and detailed description of the LAB diversity of nem chua, a Vietnamese fermented meat product, using a polyphasic approach, consisting of $(GTG)_5$-PCR fingerprinting and sequence analysis of the phenylalanyl-tRNA synthase (*pheS*) and/or RNA polymerase α subunit (*rpoA*) genes, used in combination with PCR-DGGE in order to obtain an even more complete picture of the nem chua LAB community.

The study of Wanangkarn et al. (2014) applied restriction fragment length polymorphism (RFLP) analysis to identify the LAB isolated from "mum" Thai fermented sausages during fermentation and storage, in order to facilitate the development of LAB starter cultures that enable controlled processing during mum manufacturing, and provide a more consistent and higher quality product. The authors aimed to identify the dominant LAB species and evaluate the variations in LAB community composition in Thai fermented (mum) sausages manufactured using the conventional processing method and to evaluate the feasibilities and efficiencies of restriction enzymes used for the differentiation of the LAB in RFLP analysis. Their results showed that RFLP analysis is capable of rapidly and easily differentiating and identifying the LAB isolated from mum sausages during fermentation and storage, indicating *Lb. sakei* and *Lb. plantarum* as predominant during fermentation, and marked increases of *Leuconostoc mesenteroides* during the storage. These findings also illustrated that chilled storage combined with vacuum packaging validly influenced the microbial ecology of the mum sausage.

Moreover, the dynamics of the bacterial population throughout the ripening of Galician chorizo, a traditional dry-fermented sausage produced in the north-west of Spain, were investigated by using classical

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and molecular approaches (Fonseca et al. 2013). The combination of the results obtained from microbial counts, species and genus-specific PCR as well as real-time quantitative PCR (qPCR) allowed the identification for the dominant bacterial species and the study of the variation in the community composition over the ripening period. In this study, the real-time PCR was shown to be an efficient tool for the study of the complex associations developed in meat fermentations and for the characterization of dominant populations.

At last, the viable microbial community present in Ciauscolo salami of Central Italy was profiled by ARDRA and RAPD-PCR (Federici et al. 2014). The molecular identification highlighted the presence of a high variety of LAB species confirming previous data reporting that *Lb. sakei*, *Pd. pentosaceus* and *Lb. plantarum* are the most common lactobacilli in Italian (Comi et al. 2005, Greco et al. 2005) and European (Aymerich et al. 2003) fermented meat sausages. Ciauscolo productions showed a high genotypic heterogeneity of LAB population based on both the high number of different species identified and intra-specific genotypic variation by RAPD analyses. The isolates were also examined for their potential use as probiotics, since probiotic properties are very strain-dependent. In this study, in vitro methods were selected to investigate the diversity of biological properties of LAB of food origin in comparison with LAB culture collection set. In addition, we showed that food LAB strains have probiotic abilities. In fact, isolation and characterization of novel LAB strains from not investigated niches could have the twofold advantage of revealing taxonomic characteristics and obtaining strains with interesting functional traits.

3 CONCLUSION

Understanding the dynamics, diversity and behavior of microbiota during meat products fermentation is a very interesting and challenging task. As underlined above, microorganisms present in fermented meat products possesses specific characteristics that lead to the development of typical textures and flavour, and putative health-conferring properties; so, the comprehension of the ecology of the fermentation process can help the producers to reach high quality of their products. The surprising advancements in the field of molecular biology, in the last years, revolutionized the way microorganisms are detected and characterized and allowed development of new approaches to study the microbiota without any cultivation. The information collected so far, by applying molecular methods in this field, confirm the findings that were produced by the first studies on the ecology of fermented sausages, but with a deeper level of comprehension.

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DGGE approach allows to compare different products and understand how similar they are, based on the profile of the populations that are detected, moreover the molecular characterization of isolates during the transformation process permits to understand species and strains biodiversity.Recently, the knowledge achieved for the study of the microbiota of fermented meat products is substantial, with a great step forward regard to the investigation of strain dynamics and successions.

The outcomes from the first studies on this specific subject, underline the complexity of the ecology at strain level, during sausage fermentation; biotypes of the same species are coming from the raw materials and the processing plants and their ability to grow and dominate depends on a lot of parameters, as microbial ecosystem is characterized by a species-site-dominance, and this dominance is closely related to the environmental parameters. In fact, traditional fermented sausages show a distinctive organoleptic profile that can be explained by admitting that the isolated strains, although they belong to the species commonly regarded as responsible for sausages fermentation, they possess specific physiological and technological characteristics that make these traditional products unique. Therefore, microbial populations affect significantly the organoleptic and sensory characteristics of the final product by specific and important metabolic activities that contribute to improve quality and safety of fermented sausages.

The possibility to follow specific strains nowadays is possible through the application of molecular methods for the characterization, even if cultivation and isolation are still necessary. However, protocols for direct characterization, using total DNA extracted from the sample, are now available; the application of NGS techniques contribute to describe microorganisms, their activities and dynamics and their role in changes of the quality of these products that is strictly connected to the populations able to develop and to carry out the transformation process, and more specifically to certain biotypes within a species. These techniques able to profile complex microbial ecosystems; if regions of the DNA able to differentiate between strains of the same species are properly selected, their diversity in terms of abundance of a specific sequence can be evidenced, while targeting the RNA, important insights of specific activities of the different biotypes can be obtained. This aspect is extremely relevant, when the differences at the sensory profile, between sausages produced with the same ingredients but in plants of different geographical areas, are taken into consideration, and also because, in recent times, microbial species used in fermented meat products are gaining increasing attention in the area of probiotics. For this reason, the isolation and characterization of microbial species from un-investigated niches of novel fermented meat products are spreading

as they could have the two-fold advantage of revealing taxonomic characteristics and obtaining strains with interesting functional traits that can be used in technological and/or probiotic applications as live microbial feed supplements that beneficially affect the health.

Key words**:** *culture-dependent and culture-independent molecular methods, Denaturing Gradient Gel Electrophoresis (DGGE), Next Generation Sequencing (NGS), identification and characterization, dynamics and changes of microbial ecology.*

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