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MICROBIOTA OF FERMENTED MEATS: MICROBIAL CHARACTERIZATION AND CURRENT INVESTIGATIONS

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This contribute is about the diversity of fermented meat microbiota, with a focus on traditional Mediterranean products, such as fermented sausages. The diversity of 'technological' microbiota, that is, of microbiota which contributes to improve meat product sensory qualities, such as Lactic Acid Bacteria (LAB) and coagulase-negative cocci CNC), is highlighted. The criteria to select appropriate starter cultures are also discussed. Furthermore, the importance to develop indigenous starters is treated. In fact, such bacteria may make it possible to produce several typical regional fermented meat products with specific flavors. The need to improve the knowledge about their microbiota is highlighted. Consequently, the necessity to use innovative approaches based on Next Generation Sequencing (NGS) technologies, which allow a better understanding of bacterial physiology and of transformation processes dynamics, is underlined. 16S rDNA sequencing based profile would provide a broad overview of the microbial composition of a food matrix, but this technique lacks the necessary resolution to provide a depth species identification and an assessment of the microorganisms functional capability. For this reason, metagenomics and metatranscriptomics would be useful. Current investigations about the fermented meat microbiota mainly based on the most innovative methods approaches are then discussed.

Keywords: Fermented meat, Microbiota, Starter culture, Lactic acid bacteria, Coagulase-negative cocci, NGS techniques

INTRODUCTION

Meat can be easily contaminated by pathogenic microorganisms present in animals prior to being slaughtered. Therefore, it is relevant to make meat safe for consumers in terms of stability, transportation and storage. One of the methods adopted to achieve these aims is the meat fermentation.

The origin of cured and fermented meat products is very old. Initially, this technology was used for preservation purposes and this was its main use for centuries. It was soon discovered that the meat shelf-stable could be obtained by adding salt and subsequently drying or fermenting the meat (Gul and Wani, 2016). This last process was mainly empirical and based on experience transmitted by manufacturers from generation to generation. Later, the purpose was progressively adjusted to meet consumer' needs and to obtain a product with better sensorial characteristics. Technological progresses and improved knowledge about biochemical mechanisms related to product flavour and texture development (Toldrá, 2011) made it evident that fermentation processes involve complex microbial ecosystems and interactions including bacteria, yeasts, and moulds. At the same time, industrial development has led to the use of starter cultures to standardise and control fermented meat production.

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Therefore, we can define fermented meat products as food products consisting of meat undergoing to a natural fermentation by its own microbial flora to give it desirable features, or meat inoculated with microbial starter cultures under controlled conditions.

Figure 1 shows the meat production stages with the addition of cultures.

Even now, most local manufacturers adopt the natural fermentation process without inoculation or any other artificial alteration.

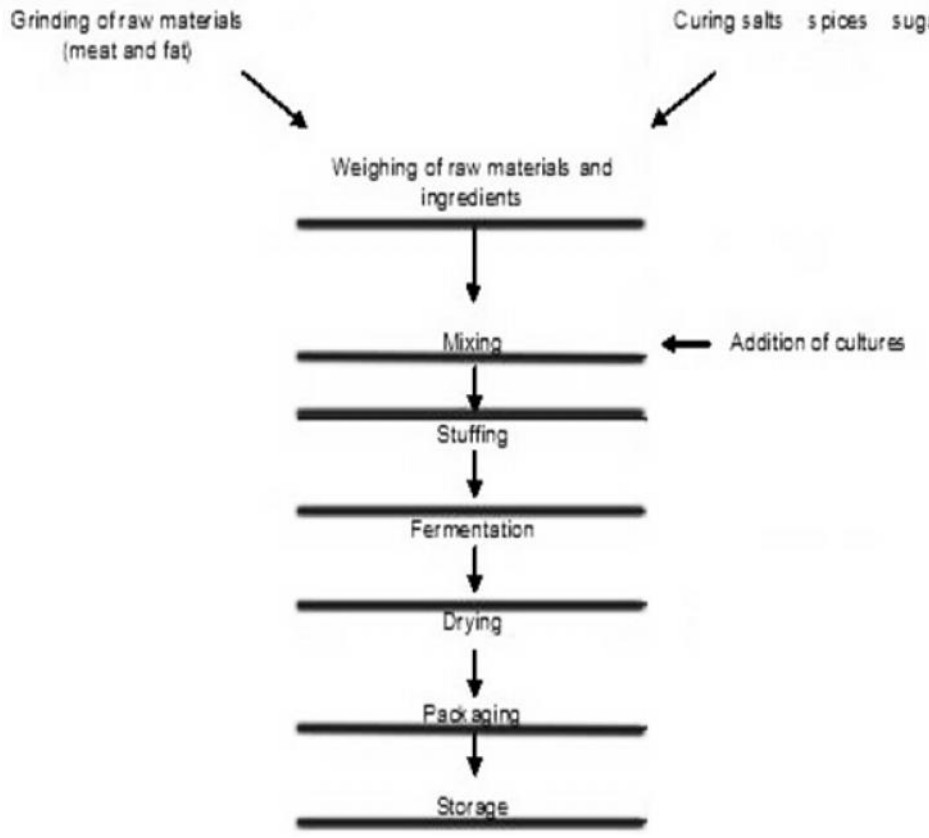
In particular, in Mediterranean regions traditional artisanal fermented meat products are widely produced. These products include salami, ham, and sausages and are very popular foods around the world, where different cultures contribute to their texture, flavour, and safety. They are highly perishable foods and a rich source of nutrients. This provides both to pathogenic and non-pathogenic microbes a suitable environment to grow during production stages and storage process.

The most important microorganisms responsible for product transformation during fermentation are Lactic Acid Bacteria (LAB) (mainly *Lactobacillus* spp.) and coagulase-negative cocci (*Staphylococcus* and*Kocuria* spp.), which seem to be able to survive during this process.

Fermented meat products are one of the most common sources of Biogenic Amines (BA). During their fermentation, maturation, and storage, suitable environmental conditions encourage the activity of microorganisms which produce decarboxylase enzymes and consequently the accumulation of BA. Besides the microorganisms' metabolic activity, BA increase depends on meat composition, additives, such as salt, sugar, nitrites, fermentation time and storage conditions. These factors strongly influence microbial growth and interactions among microbial communities, as well as acidification and proteolysis, determining the decarboxylase enzyme activity (Bover-Cid *et al.*, 2006; Latorre-Moratalla *et al.*, 2012; and Sarkadi, 2017).

LAB strains strongly inhibit the spoilage bacterial growth without affecting the organoleptic properties of the

Figure 1: Meat Production Stages with Addition of Cultures



products (Metaxopoulos *et al.*, 2002; and Aderiye *et al.*, 2006). The control of fermentation by introducing competitive LAB starter strains is an important method suggested to retard the synthesis of BA and to prevent the development of amine-producing bacteria in meat products, which leads to health benefits (Ammor *et al.*, 2007).

MICROBIOTA IN THE ENVIRONMENT OF FERMENTED MEAT PROCESSING

If the microbiota of fermented meat products is well described in the literature, the resident microbiota in the environment of the processing units is still poorly known (Talon *et al.*, 2007).

The Institut National de la Recherche Agronomique (INRA), *Centre de Clermont-Ferrand*, studied a French small-scale processing unit manufacturing sausages. The results showed that all the processing surfaces and the equipments were colonised by Coagulase-Negative Cocci (CNC) and yeasts/moulds (Chevallier *et al.*, 2006; and Morot-Bizot *et al.*, 2006). Sporadic cases of contamination of *Staphylococcus aureus* and *Listeria monocytogenes* have been detected.

The variability of the residual contamination depended on the different cleaning, disinfecting and manufacturing practices of the small-scale processing units. The inadequate cleaning of equipment pieces has often been identified as a cause of the growth of pathogens. Many studies investigated the pathogen flora of food processing environments such as *L. monocytogenes* in pork and poultry processing plants and products (Chasseignaux *et al.*, 2002) and *Salmonella* species in pork slaughter and cutting plants (Giovannacci *et al.*, 2001).

FERMENTED MEAT MICROBIOTA

The microbiota of fermented meat products plays a key role in giving them their final features. Microbial ecology varies according to the product and several factors. In fact, the prevalence of one or more microbial groups doesn't just depend on the quality of the raw material, but also on the adopted technique.

In order to describe the microbiota of fermented meat products, we will refer to the one of traditional fermented sausages, composed by a mixture of meat (often pork), pork fat in variable ratio and salt eventually including sugar, nitrate and/or nitrite.

The indigenous microbiota, which can come from the raw materials or from the manufacturing environment, is composed of useful microorganisms (CNC, LAB, yeasts/moulds) for the fermentation and final flavour of sausages, but also spoilage bacteria (Pseudomonas, Enterobacteria) and Enterococci. It depends on the diversity of formulation, fermentation and ripening practices, which can be very different in terms of temperature, duration and relative humidity (Lebert *et al.*, 2007). However, despite the different practises within the countries, or the regions, the microbial populations showed similar evolutions.

In traditional sausages, LAB constitutes the dominant microbiota at the end of the ripening phase. The LAB initial population is usually low in the raw material (3-4 log cfu/g), but it becomes dominant during the fermentation stage and remains constant during ripening at 7-9 log cfu/g (Cocolin *et al.*, 2001; and Comi *et al.*, 2005). LAB growth is often correlated with the decrease in pH in the first stage of product maturation.

CNC has a relevant population at the end of ripening (6-8 log cfu/g), while its initial level varies from 3.1 to 4.4 log cfu/g.

LAB and *Staphylococcus* or *Kocuria* are considered as technological microbiota, because they help to improve meat product sensory qualities. LAB provide a rapid acidification, *Staphylococcu*s and *Kocuria* improve colour and flavour.

Among lactobacilli, *Lactobacillus sakei*, *Lactobacillus curvatus*, and/or *Lactobacillus plantarum* generally constitute the predominant microbiota during traditional fermented meat ripening. *L. sakei* is often the dominant one and can represent more than 42% of the isolates.

L. curvatus is the second species identified; it is dominant in some Greek or Italian sausages (Comi *et al.*, 2005; Rantsiou *et al.*, 2005; and Drosinos *et al.*, 2005). *L. plantarum* is the third one; it dominates the LAB flora in a Greek sausage. Many other LAB are identified but represent a minor population (*L. alimentarius, L. casei, L. delbrueckii, L. farciminis, L. paraplantarum, L. pentosus and L. sharpeae*).

Staphylococcus xylosus is the most common species in Greek, Italian and Spanish traditional products in the ripening stage (Cocolin *et al.*, 2001; and Blaiotta *et al.*, 2004).

STARTER CULTURES

Meat starter cultures are facultative hetero fermentative strains of micro-organisms which form **lactic acid** from hexose sugars, for instance glucose and lactose as their

metabolic products. They also produce acetic acid whose amount is typically 1/10 of the lactic acid quantity. Therefore, the increase of the acidification in the fermented meat products is primarily due to the LAB along with the micrococci and/or coagulase-negative staphylococci, responsible for the improvement of colour and aroma due to their proteolytic and lipolytic actions.

Increased yields of bioactive peptides (organic substances formed by amino acids joined by covalent bonds known as amide or peptide bonds) in fermented foods may be achieved by the selection of high proteolytic microorganisms and the suitable combination of co-cultures. Bioactive peptides have potential health benefits on cardiovascular, nervous, gastrointestinal and immune systems (Martinez-Villaluenga *et al*., 2017).

Most LAB meat starter cultures belong to the species *Lactobacillus pentosus, L. casei L. curvetus, L. planterum, L. sakei, Pediococcus acidilactici, P. pentosaceus* (Singh *et al.*, 2012).

The selection of appropriate starter cultures with amino oxidase activity is essential for preventing the development of high levels of biogenic amines in fermented meat products (Suzzi and Gardini, 2003). LAB inability to produce BA and their capability to grow well at a given temperature are fundamental criteria for their selection. The production of bacteriocins (antibacterial peptides) also enhance LAB competitiveness against pathogens (Somda *et al.*, 2011).

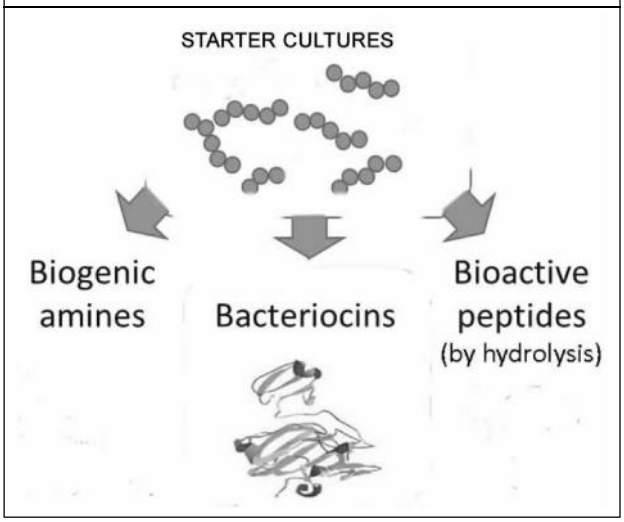
The application of this produced antimicrobial compounds as a natural barrier against pathogens and food spoilage has been proven to be efficient (Zacharof and Lovitt, 2012).

Furthermore, starter cultures capable to compete among non-starter bacteria during the ripening and storage can further avoid BA production.

Figure 2 shows desirable and undesirable bioactive metabolites produced during fermentation process impacting starter cultures choice.

However, nowadays, the development of indigenous starters is taking a relevant role. In fact, it may contribute to a diversification of the market that thus will be able to produce several typical regional fermented meat products with specific flavours. Furthermore, these kind of starters, which are able to dominate the microbiota of products, do not seem to modify the initial flavour and are well adapted to the meat environment as well as to the specific manufacturing process.

Figure 2: Desirable and Undesirable Bioactive Metabolites Produced During Fermentation Process



CURRENT FERMENTED MEAT MICROBIOTA INVESTIGATIONS: A SUMMARY

A very large number of investigations concerned the study of fermented meat microbiota and its dynamics. These studies have been conducted on several kinds of fermented meat, coming from different countries and by adopting different technological approaches.

An integrated method, based on culture-dependent and independent methods, has been applied (Aquilanti *et al.*, 2007)], in which the bacteria and yeast of a salami produced in Central Italy have been investigated. The viable counts revealed a dominance of LAB over Coagulase Negative Cocci (CNC) and yeasts. From the molecular identification of the isolates, the prevalence of *Lactobacillus curvatus, Lb. plantarum* and *Staphylococcus xylosus* was shown among the bacteria, while *Debaryomyces hansenii* was species among the yeasts.

In another study (Villani *et al.*, 2007) was investigated the microbial ecology of a traditional dry fermented sausage from southern Italy. The ripened fermented sausages were characterized by high microbial loads of both staphylococci and lactobacilli. Selected strains of *S. xylosus, L. sakei,* and *L. curvatus* were characterized for their technological properties in the ripening conditions of the fermented sausages in order to choose an autochthonous starter formulation.

The complex ecology of dry fermented sausages has been also investigated by adopting an approach based on the analysis of the Denaturing Gradient Gel Electrophoresis (DGGE) (Cocolin *et al.*, 2011). In this study it is highlighted that two main species of LAB, namely *Lactobacillus sakei* and*Lb. curvatus* are involved in the transformation process and that they are accompanied by *Staphylococcus xylosus*. The DGGE protocol was also used to monitor the dynamic changes in the microbial population during ripening of natural Italian fermented sausages. In this study LAB were individuated together with other organisms, mainly members of the family *Micrococcaceae* and meat contaminants, such as *Brochothrix thermosphacta* and *Enterococcus* sp., during the first 3 days of fermentation. After 3 days, LAB represented the main population, which was responsible for the acidification and proteolysis determining the characteristic organoleptic profile of samples. These findings were generally confirmed in different regions of the world, mainly in the Mediterranean countries where dry fermented sausages have a long tradition and history.

Furthermore the bacterial biodiversity during the maturation process of three traditional sausages produced in the North of Italy was investigated by adopting culture-dependent and culture-independent methods (Cocolin *et al.*, 2009). By using plate counts, microbiota was dominated by LAB, with minor contribution of CNC and yeasts. The species more frequently isolated were *Lactobacillus sakei* and *Lactobacillus curvatus.*

In another investigation (Gazzola *et al.*, 2012) a sausage model was used to evaluate the persistence of antibiotic resistant enterococci during meat fermentation and to assess horizontal gene transfer among bacteria involved in meat fermentation. By means of combined techniques, transconjugant strains carrying both tetracycline and erythromycin resistance genes were identified in enterococci, pediococci, lactobacilli and staphylococci groups. These results suggest that the sausage model provides a suitable environment for horizontal transfer of conjugative plasmids and antibiotic resistance genes among food microbiota.

A relevant review about the application of molecular methods for the identification and characterization of strains from fermented products underlines the need to follow in more depth dynamics of the transformation process. Many studies highlighted and used Next Generation Sequencing (NGS) techniques in order to investigate the microbiome of fermented foods (more than 60%), while the utilization of these techniques in studying the microbiome of non-fermented foods was limited.

A large number of these studies used 16S rDNA sequencing approach to analyze food microbioma (Yu *et al.*, 2017) and fermented meats (De Filippis *et al.*, 2017). An initial 16S rDNA sequencing based profile would provide a broad overview of the microbial composition of a food matrix.

However, this technique lacks the necessary resolution for providing a depth species identification and an assessment of the microorganisms functional capability. For this reason, in the last decade, metagenomics and metatranscriptomics approaches were more often used for food microbiome characterizations and their usage is gradually growing.

The utility of NGS techniques in fermented foods was extensively reported. Traditionally, most NGS related food microbiome studies have focussed on fermented foods, such as cheese, kimchi and sausages (Patra *et al.*, 2016; and De Filippis *et al.*, 2017). Different studies characterized the microbial composition of fermented foods as well as identified the changes in microbial structure over time, along with changes in the gene expression patterns related to different fermentation steps (Bokulich and Mills, 2012; Jung *et al.*, 2013; Ahn *et al.*, 2014; and Lessard *et al.*, 2014). A dry fermented sausage, that is typical of a regional area of Northern Italy, has been studied by the application of NGS techniques, coupled to the PCR amplification of the 16S rRNA subunit (Polka *et al.*, 2014). Thirty-two different *Staphylococcus* and 33 *Lactobacillus* species where identified in the salami coming from several producers. Multivariate analyses also showed that batches from 6 local producers tend to cluster altogether after 21 days of ripening, and thus indicating that NGS has the potential for fine scale differentiation of local fermented foods. Finally, by using metagenomic approaches, the addition of NaCl to meat improve texture, flavour, taste and also to extend shelf life has been analyzed (Fougy *et al.*, 2016). Improvements in sausage meat processing with higher salt concentrations combined with vacuum packaging increased the abundance of a subpopulation consisting of *Enterobacteriaceae*, *Enterococcaceae* and *Leuconostocaceae* families, which also helped to delay spoilage.

CONCLUSIONS AND DISCUSSION

This work underlines the microbiota diversity of fermented meat products and its relevant impact in fermentation. In order to better characterize such complex process the use of molecular methods based on NGS technologies is appropriate to provide a deeper microbiota analysis. These approaches for the identification and characterization of isolated strains allow to understand the dynamics of the transformation processes. Furthermore, NGS, which contribute to food investigation advances by producing a larger volume of data at a decreasing price, has a great advantage over more traditional culture independent analysis methods, in which only a fraction of the microbiota of the sample can be identified. In fact, in NGS a massive quantity of sequences are generated from a single sequencing run, with the possibility of obtaining a large amount of information in a relatively short time (Koboldt *et al.*, 2013).

Finally, the integration of different methods, such as, for example, approaches based on chemical analysis and metagenomics approach, is appropriate for studying fermented meat microbiota. In fact, the chemical analysis allows to evaluate pH, the occurrence of organic acids, biogenic amines and the prevalent volatile compounds (VOCs). The metagenomic analysis allows a comprehensive picture of the bacteria present in the samples. If the shotgun technique (a metagenomic technique for sequencing the entire genome of a given organism) is used, this allows to highlight changes in the evolution of microbial composition of foods, in contrast to the amplicon sequence method that typically is much more suitable for bacterial identification.

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