Sausage fermentation and starter cultures in the era of molecular biology methods

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Highlights

- Starter cultures in fermented sausages are mixtures of lactobacilli, pediococci and coagulase negative cocci
- Their correct use guarantees improved safety and quality
- The ecology of fermented sausage is very complex and starters have to be able to compete with native microbiota
- *Lactobacillus*, *Staphylococcus, Debaryomyces* and *Penicillium* are the main genera involved in sausage fermentation
- Next generation sequencing approaches allow for more in depth studies of the microbial ecology and functionality during fermentation

Abstract

Fermented sausages have a long tradition originating from Europe and they constitute a significant part of the Mediterranean diet. This kind of products has a specific microbiota that is typical of the region or area where they are produced. Therefore, in order to protect the traditional aspect of these products, it is essential to understand the microbial ecology during fermentation by studying the dynamic changes that occur and to select autochthonous starter cultures that can be used in the production. In this paper we summarize the state of the art concerning the selection and use of starter cultures and ecology aspects of naturally fermented sausages. We pay particular attention to the application of bacteriocinogenic strains as they could provide an additional tool in the prevention of foodborne pathogens as well as enhancing the competitiveness of the starter organisms. Microbial ecology of fermented sausages has been determined by traditional microbiological methods, but the introduction in food microbiology of new molecular techniques complements the studies carried out so far and allows scientists to overcome the limitations of traditional methods. Next Generation Sequencing (NGS) techniques represent a change in the way microbiologists address ecology and diversity in foods. Indeed the application of metataxonomics and metagenomics will permit a detailed understanding of microbial ecology. A thorough knowledge of the mechanisms behind the biological processes will enhance meat fermentation control and modulation to obtain products with desired organoleptic properties.

Keywords: Lactic Acid Bacteria; Coagulase Negative Cocci; Starter Cultures; Bioprotection; Ecology; Metataxonomics; Metagenomics

1. Introduction

In Europe, dry fermented sausages have a long tradition originating from Mediterranean countries during Roman times. Processing conditions, as well as ingredients and additives, vary among the different types of fermented sausages (Gardini *et al.*, 2001). In fact, 'typical' foods of any region or area have their own peculiar characteristics that are deeply rooted in tradition and linked to the territory and which arise from the use of local ingredients and specific production techniques (Aquilanti *et al.*, 2007; Casaburi *et al.*, 2007). The production process begins with small pieces of meat and fat that are minced; salt and spices and in some cases sugar, herbs and/or other ingredients are then added. The homogenised mixture is then stuffed into casings, and undergoes fermentation and drying. European legislation, under Reg. EC 1333/2008 (and subsequent modifications), allows the use of nitrate and nitrite as preservatives, unless subject to other regulations for protected denomination of origin (PDO) products (Aquilanti *et al.*, 2016). The qualitative characteristics of fermented sausages are known to be largely dependent on the quality of the ingredients and raw materials, the specific conditions of the processing and ripening, and the composition of the microbial population (Aquilanti *et al.*, 2007). Pathogenic and spoilage bacteria are inhibited; consequently, the final product has an increased shelf-life (Hugas and Monfort, 1997).

Meat fermentations are complex microbial ecosystems in which bacteria, yeasts and molds coexist. Considerable microbial diversity is observed during the fermentation process and is evidenced by the presence of several species belonging to different genera, but also strains of the same species. Through fermentation, highly perishable raw materials, such as meat and fat, are transformed in microbiologically stable final products, characterized by a defined sensory profile, enhanced due to sodium chloride supplementation and to the drying process (Cocolin *et al.*, 2011). Changes that occur during fermentation and drying influence the aroma development in fermented sausages (Flores *et al.*, 2004).

Many typical fermented meat products are still produced with traditional technologies without selected starters. However, in the modern sausage production, the use of starter cultures is becoming more frequent to guarantee safety and to standardize product properties, for example consistent flavor and color and shorter ripening time (Cocolin *et al.*, 2001).

2. Starter cultures

Starter cultures are preparations that contain actively growing or resting forms of microorganisms that with their metabolic activity (Fig. 1) impart desired effects during fermentation (Hammes and Hertel, 1998). Industrialized production of starter cultures is a consequence of the gradual shift in sausage production from small local producers to large-scale processing plants and the increasing awareness of the risks for consumer health, in view of overall process efficiency (Magistà *et al.*, 2017). The introduction of starter cultures has become essential in order to shorten the ripening period, ensure colour development, enhance the flavour and improve product safety, given that industrial production of fermented sausages is increasing (Lücke, 1986). In fact, a starter culture should be capable of conducting the fermentation, colonizing the product and dominating over other microorganisms from the beginning to the end of the process (Cocolin *et al.*, 2006).

On the other hand, the use of commercially available starters, mainly constituted of lactic acid bacteria and coagulase negative cocci, may result in a loss of peculiar organoleptic characteristics found in spontaneously fermented sausages with an impoverishment of flavor and aroma. For this reason, in several European countries, the artisanal sausages that are manufactured by relying on an unknown 'factory biota' are preferred by the consumer (Samelis *et al.*, 1994). The quality of such artisanal, spontaneously fermented sausages possess distinctive characteristics and are often superior if compared to controlled fermentations, inoculated with industrial starters. The principal differences between traditional and industrial fermented product are summarized in the table 1.

This is due to the technology used, the properties of the raw material (Moretti *et al.*, 2004) and the specific composition of the microbiota (Leroy *et al.*, 2006). Nonetheless, Sunesen and Stahnke (2003) reported that sausages produced with commercial molds show more consistent flavor, taste, drying rate, and a more uniform appearance with respect to artisan ally fermented sausages.

The microbial ecology of fermented sausages has become of increasing interest over the last few decades given that different genera, species, and even strains, have been shown to significantly affect the sensory traits of fermented sausages (Rantsiou and Cocolin, 2006). Production of artisanal sausages largely depends on the skill and experience of the meat manufacturer and may be considered an art rather than a process fully based on scientific and technological understanding. Meat fermentation is, in fact, a complex biological phenomenon accelerated by the desirable action of certain microbes in the presence of a variety of

97 synergistically acting or competing species. A great variability in the quality of the products is due to
98 traditional practices and variation in the microorganisms involved in the process.
99 De Vuyst (2000) underlines that it is of primordial importance to investigate and analyze the influence of the
100 environment on the performance of a starter culture before using it in a selected product.
101 In order to protect the traditional aspects of these products and to select autochthonous starter cultures to be
102 used, it is essential to understand the microbial dynamics during fermentation (Rantsiou and Cocolin, 2006).
103 Therefore, a current quest is to develop indigenous starters that guarantee hygienic quality and improve the
104 sensorial aspects of the product (Talon *et al.*, 2007). It should, however, be considered that the law allows
105 only the use of qualified presumption of safety (QPS) in the EU, or generally recognized as safe (GRAS) in
106 the US, microorganisms in food production. In Italy, only *Lactobacillus*, *Pediococcus*, *Micrococcus*,
107 *Debaryomyces*, and *Staphylococcus xylosus*, *Staphylococcus simulans* and *Staphylococcus carnosus* are
108 authorized as starters cultures for sausage production (Gazzetta Ufficiale, 1995).

109 ## 2.1 Starter culture selection parameters

110 So far the selection of a starter culture has been based on the screening of a great number of isolates in small-
111 scale food fermentations. A satisfactory performance of the selected starter culture in the process, and an
112 acceptable organoleptic evaluation of the food product are the fundamental characteristics to be found. The
113 behavior of the starter culture in relation to the environmental factors and ripening conditions encountered
114 during a specific production needs to be carefully investigated and standardized in the selection process. It is
115 necessary to understand the properties required and the specific technology and recipe for which a strain will
116 be used in order to develop the ideal starter culture (Hansen, 2002).
117 According to Holzapfel (1997), in order to improve product quality, the introduction of starter cultures in
118 traditional small-scale fermentations should incorporate considerations as (i) rapid metabolic activities (acid
119 production); (ii) improved and predictable fermentation processes; (iii) desirable sensory attributes; (iv)
120 improved safety and reduced hygienic and toxicological risks. Another important factor is the interaction in
121 mixed cultures of selected starter strains, with consideration for the behavior of these strains under defined
122 conditions, and within the food matrix. Other aspects, which should be considered, include: (i) competitive
123 behavior, viability and survival; (ii) antagonism against pathogens and spoilage microbes; (iv) rate of acid
124 production; (v) organoleptic changes; (vi) primary metabolites of fermentation; (vii) degradation of

antinutritive factors; (vii) detoxification; (viii) probiotic features (Holzapfel, 1997). Modern approaches for selection of the best strain(s) for a process integrate also technical safety and health-promoting features (Holzapfel, 2002).

It is essential to know the autochthonous microbiota of fermented food that is to be analyzed because commercial starter cultures usually originate either from substrates or from the processes in which they are applied. Factors that can contribute to the selection of microbial populations typical of a fermentation process are environmental conditions, back-slopping, adaptation and the repeated use of specific tools (Holzapfel, 1997).

3. Ecological aspects of sausage fermentation

Most European fermented sausages still follow the traditional procedures in which fermentation and ripening depend on the activities of heterogeneous microbial communities (Gardini *et al.*, 2001, Cocolin *et al.*, 2006). Two wide groups of bacteria largely predominate, lactic acid bacteria (LAB) and the group known as either coagulase–negative cocci (CNC) or (gram-positive)-catalase-positive cocci (GCC+/CPC), which includes both micrococci and coagulase–negative staphylococci (CNS) (Aquilanti *et al.*, 2016). Yeasts and filamentous fungi also play a relevant role, through the formation of a superficial film which exerts a protective action against both excessive dehydration and the oxidation of the lipid fraction due to oxygen and light (Gardini *et al.*, 2001, Cocolin *et al.*, 2006). The acidification process that is the result of the fermentation of the sugars into lactic acid by LAB, plays a fundamental role to prevent spoilage and pathogen outgrowth. Coagulase-negative cocci are involved in the proteolytic and lipolytic processes thus playing a central role in the formation of the final organoleptic characteristics (Hammes and Hertel, 1998), and contributing to nitrate reduction and color formation, as well as to prevention of rancidity. In addition, the characteristic flavors and surface aspect are due to yeasts and molds (Cocolin *et al.*, 2006). LAB are more numerous than CNC during fermentation and ripening, remaining more stable in the ripened products. Within LAB, facultatively heterofermentative lactobacilli generally prevail and, among them, the two psychrotrophic species *Lactobacillus sakei* and *Lactobacillus curvatus* are dominant. Within CNC, *Staphylococcus xylosus* neatly dominates (Aquilanti *et al.*, 2016).

The selection of specific populations adapted to a specific environment depends on ingredient composition, fermentation and maturation factors (Rantsiou *et al.*, 2005a). The production environment may have an impact on the microbial ecology of the product. For example, Greppi *et al.* (2015) showed that strains isolated from environmental samples were also detected either in the raw materials or in the product. This finding highlights that microorganisms may enter in the production plant with the raw materials. Furthermore, it underlines that the production environment is a source of continuous "inoculation", during fermentation and ripening, with strains that may have important technological characteristics and influence the characteristics of the final product.

3.1 Lactic acid bacteria

The term lactic acid bacteria is used to define a large and diverse group of microorganisms. LAB may be described as a group of Gram-positive, non-spore-forming cocci and rods, microaerophilic or facultative anaerobes, that they produce lactic acid as the major end-product during fermentation of carbohydrates (Halàsz, 2009).

L. sakei, *L. curvatus* and *L. plantarum* are the principal species of LAB usually found in meat and meat products, including fermented sausages made with different production process (Hugas *et al.*, 1993; Kittisakulnam *et al.*, 2017; Pisacane *et al.*, 2015). *L. sakei* is often isolated with the higher frequency with respect to *L. curvatus*, although sometimes the opposite occurs, or they are found at similar levels; *L. plantarum* is generally isolated with less frequency, but even in this case exceptions are found, probably due to particular processing conditions. The same is also true for the members of other LAB genera, such as *Weissella, Leuconostoc, Lactococcus*, and *Pediococcus* since they are in general found as minority species (Aquilanti *et al.*, 2016).

The genus *Pediococcus,* at the current time, consists of 13 species (Haakensen *et al.*, 2009). *Pediococcus pentosaceus* and *Pediococcus acidilactici* are the main species used in i) pediocin production, ii) fermentation processes as a starter (co-culture) to avoid contamination, and iii) probiotic supplements for animals and humans. *P. pentosaceus* cells are spherical arranged in tetrads. They are homofermentative, i.e. produce lactic acid as sole product of hexose fermentation (Porto *et al.*, 2017).

178 Lactobacilli and pediococci are the dominant microorganisms in sausages with a short ripening time from
179 early stages to the end of the process: this type of product has an acid flavor with little aroma. In contrast,
180 sausages with longer maturation times contain higher numbers of lactobacilli (Demeyer *et al*., 1986). Several
181 studies have been conducted, employing molecular methods for species and strain identification, in order to
182 understand the diversity and dynamics of LAB populations during fermented sausages production with long
183 maturation times. Rantsiou *et al.* (2005a) studied the dynamics of LAB populations involved in the process
184 of traditional fermentations performed in three countries: Hungary, Italy and Greece. In this study, 14
185 different species of LAB were detected. The only common species for Greek, Hungarian and Italian sausages
186 were *L. plantarum, L. curvatus* and *L. sakei*. Furthermore, molecular characterization of the isolates revealed
187 a country-specific geographic distribution of LAB populations... In Pisacane *et al.* (2015) the production of
188 Salame Mantovano using two different types of natural casing, deriving from two different portions of the
189 pig's intestine, was studied. Community dynamics suggested that the predominant LAB species in the two
190 types of sausages were the same.
191 Aquilanti *et al.* (2016) summarized the studies concerning the structure of LAB in Mediterranean (Northern,
192 Central and Southern Italy, Greece, France, Spain and Portugal) traditional fermented sausages. Within the
193 LAB population, facultatively heterofermentative lactobacilli generally prevailed and, among them, *L. sakei*
194 and *L. curvatus* were found to be dominant in most studies. Authors underlined that there was low species
195 variability between products of the different countries (Aquilanti *et al.* 2016).
196

197 ***3.2 Coagulase–Negative Cocci***

198 *Staphylococcus* and *Kocuria* are the most representative genera of the Gram-positive Catalase-positive Cocci
199 (GCC+) group (Morot-Bizot *et al*., 2006). The characteristic microbiota in sausages is composed of *S.*
200 *xylosus, S. saprophyticus* and *S. equorum*, but many other species have been identified such as *S. succinus, S.*
201 *warneri, S. vitulinus, S. pasteuri, S. epidermidis, S. lentus and S. haemolyticus* (Cocolin *et al.,* 2001; Talon *et*
202 *al.*, 2011; Mainar *et al.*, 2017). *Kocuria* species are ubiquitous and are highly adapted to their ecological
203 niches (Kim *et al*., 2011). In fermented sausages *Kocuria varians* and *Kocuria kristinae* were mainly found
204 (Fischer and Schleifer, 1980); moreover, *K. varians* is often found in biofilms (Raghupathi *et al*., 2016).

Although the environmental, production plant associated microbiota, can contribute to the spoilage of the meat products, ecology of *Staphylococcus* occurring in the environment of spontaneously fermented sausages has not been thoroughly studied. In fact, they showed high capacity to colonize the surfaces, the equipment and the meat products (Morot-Bizot *et al*., 2006).

Iacumin *et al*. (2006) studied the ecology and dynamics of staphylococci in three different local meat producers in the North East of Italy. In all three fermentations the same species of CNC (*S. epidermidis, S. equorum, S. warneri, S. saprophyticus, S. xylosus, S. pasteuri*) took part, but in variable quantity and proportions. The study evidenced that the slaughterhouse can partly influence the microbial composition of meat and a correlation between the isolated *S. xylosus* strains and the specific plant of production exists. This confirms the hypothesis that selection of the microbiota takes place in a production plant, depending on temperature, humidity and ingredients and influences the final sensory aspect of the product. In a comparative evaluation of the CNC communities from sausages produced in Italy, France, Greece Spain and Portugal,The dominance of *S. xylosus* clearly emerged, with the exception of the sausage productions in Greece and France. In fact, the CNC diversity, between different countries, was generally higher than that recorded for LAB (Aquilanti *et al.,* 2016).

Finally, Quijada *et al.* (2018) analyzed five Chorizo de Leon factories in the North-West region of Castilla y Leon (Spain). The factories didn't use microbial starters and adopted similar traditional manufacturing procedures. Among the five manufactures differences in microbiota composition were observed. For the CNC, *Staphylococcus* was found in all the samples, but its distribution depended on the manufacturer. In this work again the importance of country-specific microbiota in the development of traditionally manufactured products was more evident for CNC compared to *Lactobacillus* species.

3.3 Yeasts

Spontaneous fermentations are usually characterised by the presence of yeasts, but studies on the yeast biodiversity in sausages are limited. *Debaryomyces hansenii* is the yeast species most commonly isolated according to several researches but other yeast genera have also been found, such as *Candida* spp. (Gardini *et al*., 2001). An increase in pH and a decrease in lactic acid content in the sausages can be caused by these yeasts that contribute to the characteristics of the final product (Gardini *et al*., 2001). Both *D. hansenii* and

233 *Candida utilis* initially proliferate in sausages and then slowly decline (Olesen and Stahnke, 2000). *C. utilis*
234 shows a considerable potential production of several volatile compounds, such as alcohols and esters which
235 probably derive from the amino acids isoleucine, leucine, valine and phenylalanine (Olesen and Stahnke,
236 200). On the contrary, The primary and secondary metabolism, where lipases and proteinases are key
237 enzymes, are the principal processes of these organisms and can produce the typical aroma of the products
238 (Cocolin *et al*., 2006). A yeast can be added as aroma enhancer and can also stabilise the red colour of
239 fermented sausages (Olesen and Stahnke, 2000).
240 *Debaryomyces* spp. are extremophilic, perfect, haploid yeasts that asexually reproduce by multilateral
241 budding, the pseudomycelium is absent, primitive or occasionally well developed. Heterogamous
242 conjugation is the way for the sexual reproduction. In particular, *D. hansenii* is an osmo-, alo- and
243 xerotolerant yeast (Breuer and Harms, 2006). Flores *et al.* (2004) underlined that in fermented sausages,
244 *Debaryomyces* spp. can have important effects on the generation of volatile compounds during the ripening.
245 The development of the typical aroma of the sausage was possible through the inhibition of the generation of
246 lipid oxidation products and promoting the generation of ethyl esters. When *D. hansenii* was used as a starter
247 it showed a positive effect on the development of flavour characteristics and stabilisation of the reddening
248 reaction (Gardini *et al.,* 2001).
249 Cocolin *et al.* (2006a) employed a multiphasic approach during the fermentation of a traditional sausage
250 produced in Northern Italy. To profile the dynamics of yeast communities present during the maturation
251 culture-dependent and independent methods were used. Through the molecular identification by PCR-DGGE
252 and sequencing of partial 26S rRNA encoding gene of 180 isolates, *D. hansenii* resulted to be the dominant
253 species throughout the fermentation process. With molecular characterization, *D. hansenii* isolates displayed
254 a change in their population density during the maturation process of the sausages.
255 Although the origin of the meat and the factory environment have been reported as factors that can cause
256 variations on yeast populations in fermented meat products, most of the studies point towards *D. hansenii* as
257 the most frequently and abundantly isolated yeast species (Flores *et al.,* 2015; Mendonça *et al.,* 2013).
258

3.4 Filamentous fungi

The surface of dry-cured meat is colonized by molds able to grow on different environments and substrates(Magistà *et al.,* 2017). Xerotolerant and xerophilic fungi grow preferably in an environment with low water activity (a_w) and high salt concentrations as dry-cured meats. In this kind of products, fungi have also an important role in the production process because they can lead to the development of specific flavors and aromas, due to their lipolytic and proteolytic activities (Sonjak *et al.* 2011).

The genus *Penicillium* represents the major mold population of the surface mycobiota on dry-cured meat products (Sonjak *et al*., 2011). *Penicillium* is one of the most common fungi that can grow in a diverse range of habitats, from soil to vegetation to air, indoor environments and various food products (Visagie *et al*., 2014). Important taxonomic characters of *Penicillium* are the presence of conidiophore and cleistothecium (when produced). Conidiophore branching patterns have been traditionally used in the classification of *Penicillium* (Visagie *et al*., 2014). Species of *Penicillium* have been found in fermented meat sausages to be responsible for the surface colonization, most importantly *P. nalgiovense* and to a lesser extent, *P. chrysogenum* (López-Díaz *et al.*, 2001). This layer of mold is important to the sausage since it has an antioxidative effect, protecting from development of the rancidity and keeping the colour; it gives the sausage its typical appearance because it allows the development of a positive microclimate at the surface for preventing, for example, sticky or slimy characteristic of the surface (Visagie *et al.*, 2014).

4. Bioprotection

In fermented meat, the accumulation of particular metabolites as lactic acid, acetic acid, formic acid, ethanol, ammonium, fatty acids, hydrogen peroxide, acetaldehyde and bacteriocins can inhibit the growth of pathogenic and spoilage bacteria (Hugas and Monfort, 1997). The production of antimicrobial bacteriocins that leads to a better preservation of the product is a characteristic of particular starter cultures (Cleveland *et al*., 2001). Strains of all genera of LAB have been identified as bacteriocin producers. They are important in meat microbiota composition and act against bacteria closely related to the producer organisms (Lücke, 2000). However, many lactic acid bacteria (LAB) strains produce bacteriocins that are active towards pathogens or food spoilers *in vitro*, but not *in situ*, in a meat matrix (De Vuyst, 2000).

285 Different bacteriocins produced by LAB strains could be applied in food products but, at the moment, only
286 nisin and pediocin PA-1/AcH are approved for use in food preservation (Cleveland *et al*., 2001; Barbosa *et*
287 *al*., 2017; Kęska *et al.*, 2017). The application of bacteriocins in meats and meat products is allowed in three
288 different modalities: (i) direct inoculation of bacteriocinogenic LAB strains as starter or protective cultures,
289 (ii) direct application of bacteriocins from cell free supernatant (CFS) as food additive and (iii) incorporation
290 of totally or partially purified bacteriocins into the packaging (Woraprayote *et al*., 2016). Bacteriocins
291 improve a strain's competitiveness for the nutrients during fermentation, but without reducing the growth of
292 the starter organisms towards the fortuitous microbiota. (Hugas and Monfort, 1997).
293 Many strains of *Lactococcus lactis* are able to produce Nisin A that has a wide antimicrobial spectrum
294 against Gram-positive bacteria, including staphylococci, streptococci, *Listeria* spp., bacilli, and enterococci
295 (Woraprayote *et al*., 2016). Several nisin-producing *Lact. lactis* strains isolated from fermented sausages
296 showed the potential use of lactococci in this kind of products (Castellano *et al*., 2008).
297 The effect of commercial pure nisin from *Lact. lactis* subsp. *lactis* (Sigma-Aldrich) against *List.*
298 *monocytogenes* was evaluated in Turkish fermented sausages (sucuks). All products treated with nisin,
299 showed a reduction of *List. monocytogenes* population compared to the control (Hampikyan and Ugur,
300 2007).
301 *L. sakei* and *L. curvatus* strains are able to produce sakacins, bacteriocins that show high inhibitory activities
302 towards *List. monocytogenes*. Sakacin Q, produced by *L. curvatus* ACU-1, was used by Rivas *et al*. (2014)
303 for growth control of *List. innocua.* Cooked meat was artificially inoculated on surface during chilled storage
304 and four different forms of bacteriocin applications were tested: protective culture, cell-free supernatant
305 (CFS), mixture of both protective culture and CFS and freeze-dried reconstituted CFS. In this study, the most
306 effective one to control the pathogen growth was freeze-dried reconstituted CFS. In Barbosa *et al.* (2015)
307 meat-borne strains of *L. curvatus* have been described as the main source of antimicrobial compounds:
308 curvaticins. One of the isolates exhibiting inhibitory activity against *List. monocytogenes* ATCC 7644 was
309 identified as *L. curvatus* 54M16 and studied in detail for its antimicrobial substances (Casaburi *et al*., 2016).
310 In the last decades, great number of *L. plantarum* strains that produce bacteriocins were isolated from
311 different matrices including meat (Kanatani and Oshimura, 1994). In literature, numerous small, heat-stable
312 plantaricins have been described but not completely well characterized (Todorov, 2009). Schillinger and

313 Lücke (1989) isolated, from different meat products, various bacteriocinogenic lactobacilli including *L.*
314 *plantarum.* Enan *et al.* (1996) showed, for example, that plantaricin UG1 is produced by *L. plantarum* UG1
315 isolated from dry sausage and that this compound had no effect on Gram-negative bacteria, but a variety of
316 Gram-positive bacteria were sensitive. An important control of *List. monocytogenes* growth has been
317 obtained by application of plantaricins or *L. plantarum* bacteriocin producing strains (Todorov, 2009).
318 Pediocins are biomolecules that can be synthesized by some LAB and present a broad spectrum of
319 antimicrobial activity against Gram-positive bacteria (Papagianni and Anastasiadou, 2009), among which
320 *List. monocytogenes* (Porto *et al*., 2017). Kingcha *et al.* (2012) observed a significant decrease of *List.*
321 *monocytogenes* ATCC 19115 growth in Nhan, a Thai traditional fermented pork sausage, when it was
322 inoculated with *P. pentosaceus* BCC 3772 cells. The antimicrobial activity was attributed to the production,
323 by *P. pentosaceus* BCC3772, of pediocin that shows 100% amino acid identity with the commercial pediocin
324 PA-1 isoform. A correlation was observed between anti-listerial activity and *P. pentosaceus* BCC3772
325 inoculum. In addition, the authors suggested that this strain is a suitable candidate for *Listeria* control in
326 fermented pork sausage, given that no significant changes of Nahn's organoleptic properties were observed.

327 ## 5. Direct analysis of sausages and omics approaches

328 Traditional microbiological methods, namely plate counts, isolation, and biochemical identification, have
329 been often used for ecological studies of spontaneously fermented sausages. With this approach only easily
330 culturable microorganisms can be detected, while the information about microorganisms that need elective
331 enrichments or that are in a sublethal or injured state (physiological condition) are lost (Rantsiou *et al.*,
332 2005b). In fact, traditional microbiological techniques do not give a correct view of microbial diversity (Justé
333 *et al.,* 2008; Stefanis *et al.,* 2016; Silvetti *et al.,* 2017). New approaches, that take advantage of molecular
334 methods and are applied directly in a sample (direct or culture independent approaches), have been
335 introduced in the field of food microbiology and food fermentation, allowing scientists to overcome the
336 limitations of classical methods (Rantsiou and Cocolin, 2006; De Filippis *et al.*, 2018). These studies may
337 also be able to identify sentinel microbes (essentially indicator microbes linked to various pathogens), which
338 could be incorporated into food safety plans (Cocolin *et al*., 2018). The first direct or culture-independent
339 approaches were based on fingerprinting techniques such as DGGE and have been extensively applied in

fermented sausages ecology studies (Rantsiou and Cocolin, 2006; Aquilanti *et al.*, 2016). In recent years however, food microbiota studies are based on sequencing (Cocolin and Ercolini, 2015). High-throughput sequencing (HTS) techniques, undoubtedly represent a step change in the way microbiologists address ecology and diversity in foods. Unlike traditional Sanger approach sequencing that could be performed on a single DNA molecule, in HTS mixed nucleic acid molecules from a complex ecosystem can be sequenced and therefore can lead to detailed profile of the microbial populations (identified as Operational Taxonomic Units, OTU) present (Cocolin *et al*. 2018; Zhang *et al.*, 2017). In the mid 2000s HTS technologies became ubiquitous in microbial ecology studies; in fact, these technologies have been used to monitor the dynamics of microbial communities during fermentation of different types of foodstuffs and beverages (De Filippis *et al.*, 2017; Fontana *et al.*, 2016).

Metataxonomics or amplicon sequencing, is a powerful tool that allows taxonomic characterization of microbial communities, which would have been difficult if not otherwise impossible to determine using traditional microbiological techniques. For bacteria, the common amplification target is various regions of the 16S rRNA encoding gene, while for yeasts the 26S rRNA encoding gene has been used. Large sequence databases exist for these two genes (Cocolin *et al*., 2013). On the other hand, metagenome sequencing, or shotgun metagenome sequencing, is a technique where an entire mixed microbial community DNA is fragmented, prepared into a sequencing library and sequenced. Metagenomics offers the opportunity to look beyond the presence/absence of taxonomically defined entities (i.e. specific organisms) and instead to understand the relationships between microorganisms and their activities and functionalities in a particular niche (Cocolin *et al*., 2017). To understand and characterize the composition and function of the microbiota in a food ecosystem the evolution of the massive sequencing technologies such as shotgun DNA-seq or RNA-seq can help (Ferrocino *et al*., 2018). In the field of food microbiota structure and function, the identification of enzymes, pathways and mechanisms and how these operate under specific conditions may perhaps be of superior value than determining the taxonomic composition of specific samples alone (Santiago-Rodriguez *et al.*, 2016).

DNA is a chemically stable molecule, which can be found a long time after the death of a cell, RNA is more sensitive to degradation, especially in environments, like foods, where enzymes, such as hydrolases, are present. While DNA can give a good overview of the microorganisms that are or were present in a given

368 ecosystem, it cannot provide any information on what microbes are doing regarding metabolic and spoilage
369 activities and virulence factors expression. For this reason, if the goal of the investigation is to get an insight
370 on how the microorganism is behaving, the RNA is a better option (Cocolin *et al*., 2013).
371 Analysis of RNA (transcriptomics), proteins (proteomics) and metabolites (metabolomics), preferably in an
372 integrated framework, is fundamental for a full description of microbial community inasmuch metagenomic
373 sequencing has an important limitation: it cannot directly measure the functional activity of a community
374 under a given set of conditions (Franzosa *et al*., 2015).
375 Studies have used RT-PCR-DGGE coupled with 16S rRNA-based sequencing to evaluate the diversity of
376 metabolically active microbiota during the spontaneous fermentation of sausages (Połka *et al*., 2015;
377 Rebecchi *et al.*, 2015).
378 The organoleptic characteristics of the final products are influenced by volatile organic compounds (VOCs)
379 that are produced through the breakdown of carbohydrates, proteins and lipids. Ferrocino *et al.* (2018)
380 focused on studying the microbiota development and functions in an Italian fermented sausage through a
381 shotgun DNA metagenomic approach. For the first time an integrated analysis related to volatilome profile,
382 microbiota, gene content and consumers acceptability was presented. The study displayed the evolution of
383 those pathways over time and condition. The most prominent differences during spontaneous and inoculated
384 fermentation, according to the analysis, involved key genes in particular pathways: pyruvate metabolism and
385 glycolysis. For the faster metabolic activity, confirmed by the meta-metabolomics data, in this study the
386 sensory test showed that the presence of starter cultures had a negative impact on the properties of the
387 product. These methods allowed to detect shifts in microbiota composition through the recognition of
388 changes in the microbial gene content and abundance. This is important for the study of complex and
389 dynamic microbiota of food products and for food safety both to spoilage and fraud level, especially when
390 starter cultures are used.
391

392 ## 6. Conclusions

393 The use of starter cultures in food fermentations, including sausages, has allowed for a greater level of safety
394 and quality. Undoubtedly, the addition of starter strains in the meat batter for the production of sausages
395 makes the fermentation process less prone to modifications, which are responsible for products with deviated

organoleptic properties and potentially hazardous due to the presence of foodborne pathogens not inhibited during the process. However, due to the traditional and artisanal aspects that fermented sausages often possess, spontaneous fermentations are still used and represent a common practice especially in the Mediterranean countries. Isolation and selection of new strains of well known LAB and CNC species from those traditional products represents a possible alternative to the use of commercial starter cultures, which nowadays are criticized since they may lead to flattened organoleptic characteristics.

The application of modern molecular methods, such as metataxonomics and metagenomics, in fermented sausages will permit, in the near future, the understanding in detail of the microbial ecology and functions and at the same time allow for a better comprehension of the interactions of the starter cultures with the meat microbiota during sausage production. Only after a thorough knowledge of the mechanisms behind the fermentation process in meat it will be possible its control and modulation to obtain products with desired and expected organoleptic profiles.

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Figure legenda

Table 1. Main differences between traditional and industrial fermented food products (adapted and modified from El Sheikha and Montet, 2016).

Figure 1 Summary of biochemical activities performed by principal microbial groups in fermented sausages. The most frequently isolated species of each group are nominated.

623

Traditional fermented products	Industrial fermented products
Small-scale	Large-scale
Manual	Automated
Intensive to time	Time-sensitive
Possible exposure to contaminants	Minimal exposure to contaminants
Varying quality	Constant quality
Complex sensory attributes	Less complex sensory attributes
Attention to organoleptic characteristic of the product	Safety driven operation
Shorter shelf-life	Longer shelf-life
Large undefined microbial diversity	Reduced microbial diversity
Limited use of selected microbial cultures	Extensive use of microbial cultures

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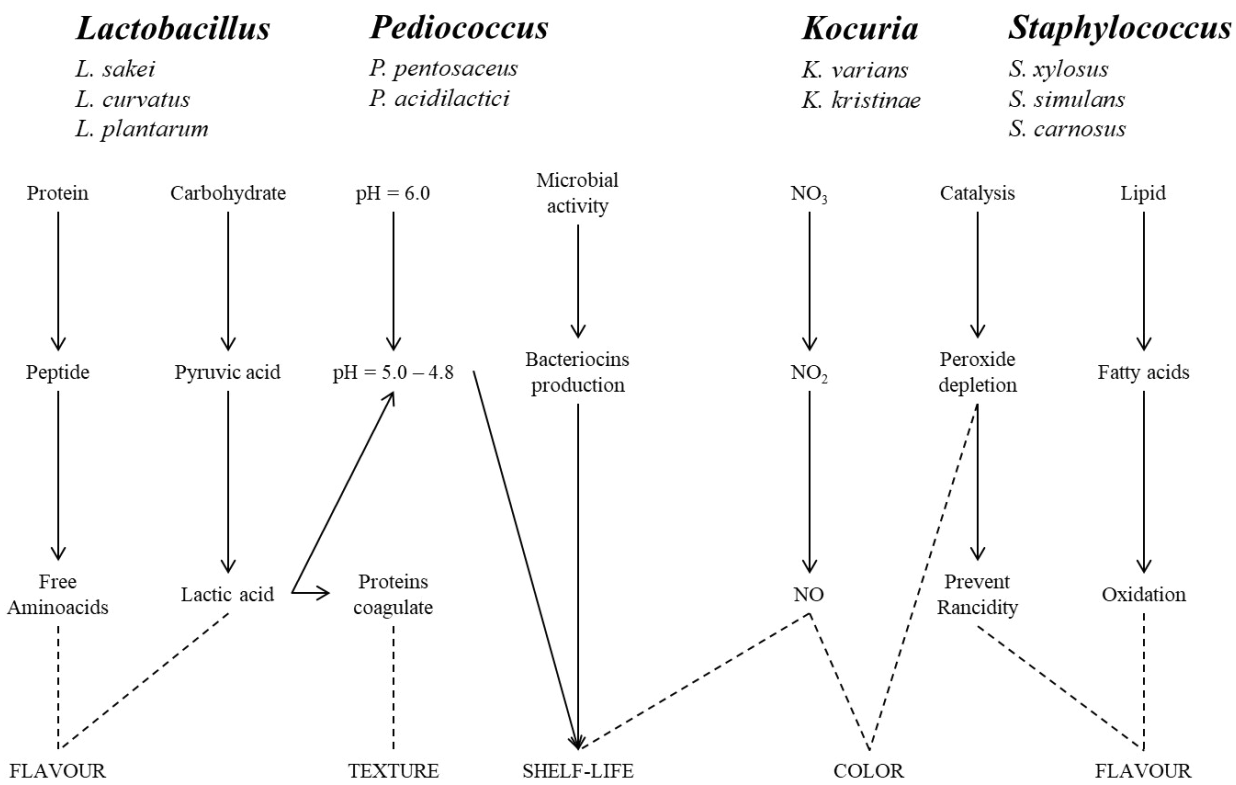

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