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# Autochthonous starter culture selection for PGI Salame Piemonte production through the microbiota analysis.

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# Chapter 1 - Introduction

Fermented meat products have an old history in human food production and consumption. Indeed, the natural activity of its microbiota was probably one of the first development in meat preservation allowing its storage and transport (Toldrá *et al*., 2014). Nowadays, the strategies used to investigate microbial communities (who is present and when?) and their associated functions (what are their respective roles?) have considerably changed and progressed providing tools to food microbiologists for conducting in-depth study of the microbiota involved in food fermentations.

In Europe, fermented meat products have a long tradition (Gardini *et al*., 2001; Talon *et al*., 2007) and it is well-established that the different processing conditions and recipes used for their preparation can have an impact on their natural microbiota (Van Reckem *et al*., 2019). In fact, their native microbiota is often composed of microorganisms originating from raw materials or from the environment (Talon *et al*., 2007). During the fermentation process, many complex biochemical changes are occurring and, since this is a delicate and complex biological system, many aspects need to be controlled to produce high-quality level products (Toldrá *et al*., 2014). Study of the microbiota that colonize these products and their impact on product organoleptic properties could help to learn how to manage these microbial resources in a better way and how to conduct the fermentation process.

In the context of artisanal food production, standardization of the manufacturing process including the use of selected starter cultures could be desirable in order to obtain safe products with constant quality attributes (flavour, texture, and colour) (Cruxen *et al*., 2019). However, on the other hand, with the use on a global scale of starter cultures provided by a small number of food culture manufacturers, such artisanal foods may lose their typicity, despite the use of traditional know-how, specific raw materials and equipment, as their microbiota is also responsible for providing them with distinct sensory characteristics when compared to similar industrial foods. The implementation in the production process of autochthonous starter cultures could be a solution.

The two bacterial groups that play a central role in meat fermentation process are lactic acid bacteria (LAB) and Gram-positive catalase-positive coagulase-negative cocci (GCC+) (Aquilanti *et al*., 2016). In particular, for fermented sausage production in Europe, the species predominantly used as starter cultures, separately or in combination, are *Latilactobacillus sakei* and *Staphylococcus xylosus* (Van Reckem *et al*., 2019). At the same time, the development of a mycobiota on the product surface is commonly observed, especially on sausage casings (Hierro et al. 2005). This mycobiota have a positive impact on fermentation and participate to the product organoleptic characteristics. The most isolated species belong to *Penicillium nalgiovense* and *Debaryomyces hansenii* (Álvarez *et al*., 2020; Iacumin *et al*., 2020).

In the last years, multi-omics approaches have been increasingly used for microbiota characterization of traditional fermented foods. Metagenomics combined with traditional microbiological methods became an essential tool to deeply understanding ecology and diversity in foods (Ferrocino *et al*., 2018; Cocolin *et al*., 2018). Indeed, such an integrative approach can be useful to get a better knowledge about this ecosystem. This

knowledge can then be used to guarantee and/or enhance food quality through desirable and autochthonous strain selection and, together with improved microbial resource management, could clearly help local producers to manufacture high quality hygienic fermented products.

### 1.1. PhD. objectives

This thesis project was part of the international project "Promotion of local Mediterranean fermented foods through a better knowledge and management of microbial resources (ProMedFoods)" which was proposed in the second axis of the call "Valorising local products through food value chains improvement" for ARIMNet2 project call 2016. ProMedFoods (2017-2021) is a project involving 7 highly specialized research teams from 6 different Mediterranean Countries (Algeria, Tunisia, France, Greece, Italy and Spain) coordinated by Professor Jérôme Mounier (France – UBO). The concept of the ProMedFoods project was to directly work with SMEs and SME associations to provide new solutions to better control fermentation processes and more efficiently manage their microbial resources. To reach this goal, local fermented food producers from each partner's country were gathered. In this context, my PhD thesis focused on PGI Salame Piemonte, an Italian dry fermented sausage.

This thesis project began with a bibliographic research that allowed me to write and publish a review (Franciosa *et al*., 2018) focused on sausage fermentation and starter cultures in the era of molecular biology methods. With this knowledge about the ecology of fermented dry sausages I acquired the competences to design the experimental setup of my PhD. project in order to reach the final aim of my PhD: selection of autochthonous starter culture for PGI Salame Piemonte production.

The first step of this work was to investigate, using culture-dependent and –independent approaches the microbiota of three spontaneously fermented batches of PGI Salame Piemonte, in order to have a complete overview about the dynamics and diversity of bacteria and fungi that characterize this specific fermented product. Bacterial and fungal microbiota were clearly different between the three batches and distinct species dynamics were observed. At the same time, a metagenomic approach was also applied in order to better unravel the microbiota dynamics and the importance of a strain-level study for a starter culture selection. In fact, this approach linked to the volatilome analysis confirmed that the more appropriate lactic acid bacteria to use for this type of fermentation belonged to *P. pentosaceus* and *L. sakei* species.

Thanks to the culture-dependent analysis of Salame Piemonte, a large working collection of bacteria including LAB and coagulase-negative staphylococci was created. After beneficial and dominant strain characterization and selection, based on their technological and safety assessment, selected strains (two *P. pentosaceus*, two *L. sakei* and one *S. xylosus* strains) were then used for pilot-scale production of Salame Piemonte with 7 different strain combinations. Sausages were subjected to microbial safety and hygiene evaluation, metataxonomic, volatilome and consumer test analyses allowing the selection of autochthonous starter cultures for PGI Salame Piemonte.

# Chapter 2

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

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**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.

### 2.1. Dry fermented sausages

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 flavour and colour and shorter ripening time (Cocolin *et al*., 2001).

### 2.2. Starter cultures

Starter cultures are preparations that contain actively growing or resting forms of microorganisms that with their metabolic activity 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 flavour 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 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 flavour, taste, drying rate, and a more uniform appearance with respect to artisan ally fermented sausages.

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

| **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 |

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 synergistically acting or competing species. A great variability in the quality of the products is due to traditional practices and variation in the microorganisms involved in the process.

De Vuyst (2000) underlines that it is of primordial importance to investigate and analyse the influence of the environment on the performance of a starter culture before using it in a selected product.

In order to protect the traditional aspects of these products and to select autochthonous starter cultures to be used, it is essential to understand the microbial dynamics during fermentation (Rantsiou and Cocolin, 2006). Therefore, a current quest is to develop indigenous starters that guarantee hygienic quality and improve the sensorial aspects of the product (Talon *et al*., 2007). It should, however, be considered that the law allows only the use of qualified presumption of safety (QPS) in the EU, or generally recognized as safe (GRAS) in the US, microorganisms in food production. In Italy, only *Lactobacillus*, *Pediococcus*, *Micrococcus*, *Debaryomyces*, and *Staphylococcus xylosus*, *Staphylococcus simulans* and *Staphylococcus carnosus* are authorized as starters cultures for sausage production (Ministero della Sanità, 1995).

### 2.2.1. Starter culture selection parameters

So far the selection of a starter culture has been based on the screening of a great number of isolates in small-scale food fermentations. A satisfactory performance of the selected starter culture in the process, and an acceptable organoleptic evaluation of the food product are the fundamental characteristics to be found. The behaviour of the starter culture in relation to the environmental factors and ripening conditions encountered during a specific production needs to be carefully investigated and standardized in the selection process. It is necessary to understand the properties required and the specific technology and recipe for which a strain will be used in order to develop the ideal starter culture (Hansen, 2002).

According to Holzapfel (1997), in order to improve product quality, the introduction of starter cultures in traditional small-scale fermentations should incorporate considerations as (i) rapid metabolic activities (acid production); (ii) improved and predictable fermentation processes; (iii) desirable sensory attributes; (iv) improved safety and reduced hygienic and toxicological risks. Another important factor is the interaction in mixed cultures of selected starter strains, with consideration for the behaviour of these strains under defined conditions, and within the food matrix. Other aspects, which should be considered, include: (i) competitive behaviour, viability and survival; (ii) antagonism against pathogens and spoilage microbes; (iv) rate of acid 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 analysed 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).

### 2.3. 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 (Cocolin *et al*., 2006; Gardini *et al*., 2001). 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 (Cocolin *et al*., 2006; Gardini *et al*., 2001). 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 colour formation, as well as to prevention of rancidity. In addition, the characteristic flavours and surface aspect are due to yeasts and molds (Cocolin *et al*., 2006). LAB are more numerous than CNS 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 *Latilactobacillus sakei* and *Latilactobacillus curvatus* are dominant. Within CNS, *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.

### 2.3.1 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 (Halász, 2009).

*L. sakei*, *L. curvatus* and *Lpb. 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; *Lpb. 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).

Lactobacilli and Pediococci are the dominant microorganisms in sausages with a short ripening time from early stages to the end of the process: this type of product has an acid flavour with little aroma. In contrast, sausages with longer maturation times contain higher numbers of lactobacilli (Demeyer *et al*., 1986). Several studies have been conducted, employing molecular methods for species and strain identification, in order to understand the diversity and dynamics of LAB populations during fermented sausages production with long maturation times. Rantsiou *et al*. (2005a) studied the dynamics of LAB populations involved in the process of traditional fermentations performed in three countries: Hungary, Italy and Greece. In this study, 14 different species of LAB were detected. The only common species for Greek, Hungarian and Italian sausages were *Lpb. plantarum*, *L. curvatus* and *L. sakei*. Furthermore, molecular characterization of the isolates revealed a country-specific geographic distribution of LAB populations. In Pisacane *et al*. (2015) the production of Salame Mantovano using two different types of natural casing, deriving from two different portions of the pig's intestine, was studied. Community dynamics suggested that the predominant LAB species in the two types of sausages were the same.

Aquilanti *et al*. (2016) summarized the studies concerning the structure of LAB in Mediterranean (Northern, Central and Southern Italy, Greece, France, Spain and Portugal) traditional fermented sausages. Within the LAB population, facultatively heterofermentative lactobacilli generally prevailed and, among them, *L. sakei* and *L. curvatus* were found to be dominant in most studies. Authors underlined that there was low species variability between products of the different countries (Aquilanti *et al*., 2016).

### 2.3.2 Coagulase–Negative Cocci

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

Iacumin *et al*. (2006a) 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 CNS (*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 CNS 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 CNS diversity, between different countries, was generally higher than that recorded for LAB (Aquilanti *et al*., 2016).

Finally, Quijada *et al*. (2018) analysed 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 CNS, *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 CNS compared to *Lactobacillus* species.

### 2.3.3. 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 *Candida utilis* initially proliferate in sausages and then slowly decline (Olesen and Stahnke, 2000). *C. utilis* shows a

considerable potential production of several volatile compounds, such as alcohols and esters which probably derive from the amino acids isoleucine, leucine, valine and phenylalanine (Olesen and Stahnke, 2000). On the contrary, the primary and secondary metabolism, where lipases and proteinases are key enzymes, are the principal processes of these organisms and can produce the typical aroma of the products (Cocolin *et al*., 2006). A yeast can be added as aroma enhancer and can also stabilise the red colour of fermented sausages (Olesen and Stahnke, 2000).

*Debaryomyces* spp. are extremophilic, perfect, haploid yeasts that asexually reproduce by multilateral budding, the pseudomycelium is absent, primitive or occasionally well developed. Heterogamous conjugation is the way for the sexual reproduction. In particular, *D. hansenii* is an osmo-, alo- and xerotolerant yeast (Breuer and Harms, 2006). Flores *et al*. (2004) underlined that in fermented sausages, *Debaryomyces* spp. can have important effects on the generation of volatile compounds during the ripening. The development of the typical aroma of the sausage was possible through the inhibition of the generation of lipid oxidation products and promoting the generation of ethyl esters. When *D. hansenii* was used as a starter it showed a positive effect on the development of flavour characteristics and stabilisation of the reddening reaction (Gardini *et al*., 2001).

Cocolin *et al*. (2006) employed a multiphasic approach during the fermentation of a traditional sausage produced in Northern Italy. To profile the dynamics of yeast communities present during the maturation culture-dependent and independent methods were used. Through the molecular identification by PCR-DGGE and sequencing of partial 26S rRNA encoding gene of 180 isolates, *D. hansenii* resulted to be the dominant species throughout the fermentation process. With molecular characterization, *D. hansenii* isolates displayed a change in their population density during the maturation process of the sausages.

Although the origin of the meat and the factory environment have been reported as factors that can cause variations on yeast populations in fermented meat products, most of the studies point towards *D. hansenii* as the most frequently and abundantly isolated yeast species (Flores *et al*., 2015; Mendonça *et al*., 2013).

### 2.3.4 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 (*aw*) 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 flavours 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).

### 2.4. 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).

Different bacteriocins produced by LAB strains could be applied in food products but, at the moment, only nisin and pediocin PA-1/AcH are approved for use in food preservation (Barbosa *et al*., 2017; Cleveland *et al*., 2001; Kęska *et al*., 2017). The application of bacteriocins in meats and meat products is allowed in three different modalities: (i) direct inoculation of bacteriocinogenic LAB strains as starter or protective cultures, (ii) direct application of bacteriocins from cell free supernatant (CFS) as food additive and (iii) incorporation of totally or partially purified bacteriocins into the packaging (Woraprayote *et al*., 2016). Bacteriocins improve a strain's competitiveness for the nutrients during fermentation, but without reducing the growth of the starter organisms towards the fortuitous microbiota (Hugas and Monfort, 1997).

Many strains of *Lactococcus lactis* are able to produce Nisin A that has a wide antimicrobial spectrum against Gram-positive bacteria, including staphylococci, streptococci, *Listeria* spp., bacilli, and enterococci (Woraprayote *et al*., 2016). Several nisin-producing *Lact. lactis* strains isolated from fermented sausages showed the potential use of lactococci in this kind of products (Castellano *et al*., 2008).

The effect of commercial pure nisin from *Lact. lactis* subsp. *lactis* (Sigma-Aldrich) against *List. monocytogenes* was evaluated in Turkish fermented sausages (sucuks). All products treated with nisin, showed a reduction of *List. monocytogenes* population compared to the control (Hampikyan and Ugur, 2007).

*L. sakei* and *L. curvatus* strains are able to produce sakacins, bacteriocins that show high inhibitory activities towards *List. monocytogenes*. Sakacin Q, produced by *L. curvatus* ACU-1, was used by Rivas *et al*. (2014) for growth control of *List. innocua*. Cooked meat was artificially inoculated on surface during chilled storage and four different forms of bacteriocin applications were tested: protective culture, cell-free supernatant (CFS), mixture of both protective culture and CFS and freeze-dried reconstituted CFS. In this study, the most effective one to control the pathogen growth was freeze-dried reconstituted CFS. In Barbosa *et al*. (2015) meat-borne strains of *L. curvatus* have been described as the main source of antimicrobial compounds: curvaticins. One of

the isolates exhibiting inhibitory activity against *List. monocytogenes* ATCC 7644 was identified as *L. curvatus* 54M16 and studied in detail for its antimicrobial substances (Casaburi *et al*., 2016).

In the last decades, great number of *Lpb. plantarum* strains that produce bacteriocins were isolated from different matrices including meat (Kanatani and Oshimura, 1994). In literature, numerous small, heat-stable plantaricins have been described but not completely well characterized (Todorov, 2009). Schillinger and Lücke (1989) isolated, from different meat products, various bacteriocinogenic lactobacilli including *Lpb. plantarum*. Enan *et a*l. (1996) showed, for example, that plantaricin UG1 is produced by *Lpb. plantarum* UG1 isolated from dry sausage and that this compound had no effect on Gram-negative bacteria, but a variety of Gram-positive bacteria were sensitive. An important control of *List. monocytogenes* growth has been obtained by application of plantaricins or *Lpb. plantarum* bacteriocin producing strains (Todorov, 2009).

Pediocins are biomolecules that can be synthesized by some LAB and present a broad spectrum of antimicrobial activity against Gram-positive bacteria (Papagianni and Anastasiadou, 2009), among which *List. monocytogenes* (Porto et al., 2017). Kingcha *et al*. (2012) observed a significant decrease of *List. monocytogenes* ATCC 19115 growth in Nhan, a Thai traditional fermented pork sausage, when it was inoculated with *P. pentosaceus* BCC3772 cells. The antimicrobial activity was attributed to the production, by *P. pentosaceus* BCC3772, of pediocin that shows 100% amino acid identity with the commercial pediocin PA-1 isoform. A correlation was observed between anti-listerial activity and *P. pentosaceus* BCC3772 inoculum. In addition, the authors suggested that this strain is a suitable candidate for *Listeria* control in fermented pork sausage, given that no significant changes of Nahn's organoleptic properties were observed.

### 2.5. Direct analysis of sausages and omics approaches

Traditional microbiological methods, namely plate counts, isolation, and biochemical identification, have been often used for ecological studies of spontaneously fermented sausages. With this approach only easily culturable microorganisms can be detected, while the information about microorganisms that need elective enrichments or that are in a sublethal or injured state (physiological condition) are lost (Rantsiou *et al*., 2005b). In fact, traditional microbiological techniques do not give a correct view of microbial diversity (Justé *et al*., 2008; Silvetti *et al*., 2017; Stefanis *et al*., 2016). New approaches, that take advantage of molecular methods and are applied directly in a sample (direct or culture independent approaches), have been introduced in the field of food microbiology and food fermentation, allowing scientists to overcome the limitations of classical methods (De Filippis *et al*., 2018; Rantsiou and Cocolin, 2006). These studies may also be able to identify sentinel microbes (essentially indicator microbes linked to various pathogens), which could be incorporated into food safety plans (Cocolin *et al*., 2018). The first direct or culture-independent approaches were based on fingerprinting techniques such as DGGE and have been extensively applied in fermented sausages ecology studies (Aquilanti *et al*., 2016; Rantsiou and Cocolin, 2006). 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*., 2018). 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 ecosystem, it cannot provide any information on what microbes are doing regarding metabolic and spoilage activities and virulence factors expression. For this reason, if the goal of the investigation is to get an insight on how the microorganism is behaving, the RNA is a better option (Cocolin *et al*., 2013).

Analysis of RNA (transcriptomics), proteins (proteomics) and metabolites (metabolomics), preferably in an integrated framework, is fundamental for a full description of microbial community inasmuch metagenomic sequencing has an important limitation: it cannot directly measure the functional activity of a community under a given set of conditions (Franzosa *et al*., 2015).

Studies have used RT-PCR-DGGE coupled with 16S rRNA-based sequencing to evaluate the diversity of metabolically active microbiota during the spontaneous fermentation of sausages (Połka *et al*., 2014; Rebecchi *et al*., 2015).

The organoleptic characteristics of the final products are influenced by volatile organic compounds (VOCs) that are produced through the breakdown of carbohydrates, proteins and lipids. Ferrocino *et al*. (2018) focused on studying the microbiota development and functions in an Italian fermented sausage through a shotgun DNA

metagenomic approach. For the first time an integrated analysis related to volatilome profile, microbiota, gene content and consumers acceptability was presented. The study displayed the evolution of those pathways over time and condition. The most prominent differences during spontaneous and inoculated fermentation, according to the analysis, involved key genes in particular pathways: pyruvate metabolism and glycolysis. For the faster metabolic activity, confirmed by the meta-metabolomics data, in this study the sensory test showed that the presence of starter cultures had a negative impact on the properties of the product. These methods allowed to detect shifts in microbiota composition through the recognition of changes in the microbial gene content and abundance. This is important for the study of complex and dynamic microbiota of food products and for food safety both to spoilage and fraud level, especially when starter cultures are used.

### 2.6. References

References of this chapter are integrated into the overall reference section (Chapter 7).

# Chapter 3

## Specific metagenomic asset drives the spontaneous fermentation of Italian sausages

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**Abstract**

Metagenomics is a powerful tool to study and understand the microbial dynamics that occur during food fermentation and allows to close the link between microbial diversity and final organoleptic characteristics. Each food matrix can be colonized by different microbes, but also by different strains of the same species. In this study, using an innovative integrated approach, we were able to show how strain–level biodiversity could influence the quality characteristics of the final product. The attention was placed on a model fermented food: Salame Piemonte, a Protected Geographical Indication (PGI) Italian fermented sausage. Three independent batches produced in February, March and May 2019 were analysed. The sausages were manufactured, following the production specification, in a local meat factory in the area of Turin (Italy) without the use of starter cultures. A pangenomic approach was applied in order to identify and evaluate the lactic acid bacteria (LAB) population driving the fermentation process. It was observed that all batches were characterized by the presence of few LAB species, namely *Pediococcus pentosaceus*, *Latilactobacillus curvatus* and *Latilactobacillus sakei*. Sausages from the different batches were different when the volatilome was taken into consideration, and a strong association between quality attributes and strains present was determined. In particular different strains of *L. sakei*, showing heterogeneity at genomic level, colonized the meat at the beginning of each production and deeply influenced the fermentation process by distinctive metabolic pathways that affected the fermentation process and the final organoleptic aspects.

### 3.1. Introduction

In the last ten years, in order to investigate and understand the microbial community structure of different fermented foods, shotgun DNA sequencing has been applied (Bora *et al*., 2016; De Filippis *et al*., 2019a; Ferrocino *et al*., 2018). Nevertheless, only few studies aimed at characterizing the microbiome of fermented foods and its link to the quality properties of the final product through a metagenomics/pangenomics approach. A better knowledge of the microbial resource is essential to characterize, track and monitor the genetic microbial diversity and the spread of genetic traits (Bora *et al*., 2016; Jung *et al*., 2011; Manghi *et al*., 2018). Differences at strain level could influence the metabolic and ecological adaptation capabilities of microbes and thereby how they could successfully occupy a wide range of habitats (Eisenbach *et al*., 2019) and have a different impact on the colonised environment. A pangenomic analysis could facilitate the comprehension of the microbial ecology in food fermentation and the explanation of peculiarities that characterize specific products by highlighting strain abundance and diversity (De Filippis *et al*., 2019b; Peng *et al*., 2018).

In this study, metagenomic DNA-seq, integrated with GC-MS analysis, was used to better understand strains distribution through in situ monitoring of metabolic pathways and microbial genes in spontaneously fermented Italian sausage. It is already known that during spontaneous fermentation the presence of *Latilactobacillus sakei* is usually associated with the presence of *Latilactobacillus curvatus*, *Lactiplantibacillus plantarum*, *Pediococcus*, *Staphylococcus xylosus* and *Staphylococcus equorum* (Greppi *et al*., 2015). These bacteria have a different role during the maturation process. Coagulase-negative Staphylococcaceae (CNS) are responsible for proteolytic and lipolytic metabolic activity on meat components, while lactic acid bacteria (LAB) for the acidification process, due to lactic acid production, while they also deliver important volatile organic compounds (acetic acid, ethanol, acetoin and pyruvic acid) (Ferrocino *et al*., 2018; Połka *et al*., 2014) through carbohydrate catabolism.

In order to discover the strain level variation an assembly-based approach is often applied. This approach uses sequence reads assembly (contigs) that can be grouped and assigned to discrete population bins (Bowers *et al*., 2017) to generate multiple whole genomes. A metagenome-assembled genomes (MAGs) phylogeny is a way through which we can show the genetic variability after the assembly, mapping and binning process (Anderson *et al*., 2003; Luo *et al*., 2015). Genetic variation is then described through single nucleotide polymorphisms (SNPs) or using genes as units of comparison (Méric *et al*., 2014; Sheppard *et al*., 2012). Different SNPs profiles represent individual strains. The major limitation is that this approach is applicable only for organisms with enough coverage to be assembled and binned, so only part of the genomes in a complex community can be explored (Anderson *et al*., 2003; Luo *et al*., 2015; Quince *et al*., 2017).

To obtain a strain-level phylogeny reconstruction an assembly-free metagenomic profiling can be done mapping the raw reads to reference genes or directly to contig bins. The principal advantage of this method is the potential to perform large scale strain-level analyses, but uncharacterized bacteria are difficult to profile (Luo *et al*., 2015; Quince *et al*., 2017¸ Quince *et al*., 2016). However, the genomes of the strains reconstructed through this approach are characterized by lower quality than those obtained directly from isolates (Segata, 2018). For this reason, it is always necessary to compare metagenomics data with culturomics in order to

confirm the level of diversity. It can be possible to obtain a complete overview of the specific microbiome that characterizes a food matrix only by a combination approach, to avoid the possibility to overestimate or underestimate part of the obtained data.

With the final goal of enriching the current knowledge of strain biodiversity and its influence on the final product quality, the present study takes as a model the ecosystem developing during spontaneous fermentation of Salame Piemonte PGI, a fermented sausage from the North West of Italy. A blended approach, employing culturomics and metagenomics, was chosen in order to better link diversity of strains with volatile profiles of the investigated fermented sausages.

The meta-omics approach adopted in this study has the capability to create a profile based on the presence or absence of the genes in order to characterize the microbial organisms at single strains' resolution (Manghi *et al*., 2018). This method was applied only seldom to a food matrix in the understanding of strain dynamics during a fermentation process. However, this approach can shed light on how the various microorganisms can influence food bioprocesses (Bora *et al*., 2016). Better knowledge is necessary to investigate how we can control and obtain the final organoleptic characteristics of the products by selecting the right consortia as starter cultures.

### 3.2. Materials and methods

#### 3.2.1. Sausages manufacturing

Salame Piemonte PGI were manufactured in a local meat factory in the area of Turin according to the production specification. The formulation used in the manufacturing included pork meat (lean from the shoulder and fat from the belly), salt (maximum 3%), pepper (maximum 0.4%), spices and aromatic plants (garlic, cloves, whole, crushed or infused with wine, nutmeg). Meat batter was then stuffed into casings, resulting in sausages of about 35 cm long and 3 kg in weight. Fermentation and ripening for 50 days were carried out in a climatic chamber (Ferrocino *et al*., 2018). Three sausage samples were obtained, without using the starter culture, after 4, 8, 15, 30 and 50 days of fermentation and analysed for each of three independent batches produced in February, March and May 2018.

#### 3.2.2. DNA extraction

At each sampling point, 3 aliquots of about 10 g from each sausage were collected from the core and individually homogenized with 90 ml of buffered peptone water (Oxoid, Milan, Italy) for 2 min in a stomacher (LAB blender 400; PBI, Italy).

One ml was collected and centrifugated in order to extract the total DNA from each sample. The total DNA from fermented sausage was extracted using the RNeasy Power Microbiome kit (QIAGEN Group) according to the manufacturer's instructions. One microliter of RNase (Illumina Inc. San Diego. CA) was added to digest RNA in the DNA samples with an incubation of 1 h at 37°C.

Whole metagenomics (150 bp paired-end reads) was performed on a NextSeq 550 Illumina machine by the Genewiz company (Leipzig, Germany).

### 3.2.3. Metagenomics analysis

Raw sequences were first mapped against the draft genome of *Sus Scrofa* using Bowtie2 in end-to-end sensitive mode. Reads were quality filtered with Solexa QA++ software (Cox *et al*., 2010) (Q<20) and by Prinseq (reads <60bp and dereplicated) (Schmieder R, 2011). The phylogenetic characterization of the shotgun sequences was achieved at species level of taxonomy by using MetaPhlAn2 (Segata *et al*., 2012) with default parameters. Assembly was performed with MetaSPAdes (Nurk *et al*., 2017) while QUAST (Gurevich *et al*., 2013) software was used for the quality check of the contigs. Genes prediction from each contig was performed by MetaGeneMark (Zhu *et al*., 2010). The sausages gene catalog was obtained using the pipeline describe by Ferrocino *et al.* (Ferrocino *et al*., 2018). Genes were concatenated and clustered, using USEARCH (Edgar, 2010), and aligned against the NCBI-NR database by BLASTn tools. Clean reads were then mapped against the annotated catalog with Bowtie2. The functional analysis against the KEGG database was conducted using MEGAN (Mitra *et al*., 2011) software. The KEGG gene count table was internally normalized in MEGAN with the function "normalized count". The rarefaction analysis was performed on selected genes with coverage and identity > 98% in MEGAN.

### 3.2.4. *De novo* extraction of strain genomes from metagenomes

MetaBat2 (Kang *et al*., 2019) software was used on contigs to reconstruct draft genomes (bins). CheckM (Parks *et al*., 2015) software was then used to evaluate completeness and contamination. Bins where then imported in Focus (Silva *et al*., 2014) for taxonomic assessment. High quality bins where then imported in PROKKA (Seemann, 2014) for gene prediction and annotation. Bins were classified following Bowers et al (2017) (Bowers *et al*., 2017) standards as: high-quality draft (>90% completeness, <5% contamination), medium-quality draft (>50% completeness, <10% contamination) or low-quality draft (<50% completeness, <10% contamination). The pangenome calculation and phylogenetic analysis of bins were obtained by Roary (Page *et al.*, 2015).

### 3.2.5. Analysis of volatile organic compounds

The volatile organic compounds (VOCs) in sausage samples were extracted using headspace (HS) solid-phase microextraction (SPME) and analysed by gas chromatography-mass spectrometry (GC/MS). All samples were analysed in triplicates. The analysis was conducted using a 20 ml vial filled with 3 g of mixed sample to which 10 µl of 2-octanol in ultrapure water (100 ppm) was added as internal standard. A headspace solid phase microextraction (HS-SPME) followed by gas chromatographic and spectrometric analysis (GC/MS) was carried out according to the SPME extraction previously described (Ferrocino *et al*., 2018) by using a GC-2010 gas chromatograph equipped with a QP-2010 Plus quadruple mass spectrometer (Shimadzu Corporation, Kyoto, Japan) and a DB-WAXETR capillary column with 30 m length, 0.25 mm internal diameter, 0.25 µm

film thickness (J&W Scientific Inc., Folsom, CA). Semiquantitative data (µg/kg) were obtained by measuring the m/z peak area of the quantifier ion of each identified compound in relation to that of the m/z ion of the added internal standard.

### 3.2.6. Microbial analysis

Sausage homogenates were used for lactic acid bacteria (LAB) counts and pH determination. Decimal dilutions in quarter-strength Ringer's solution were prepared and spread in triplicate on de Man-Rogosa-Sharpe (MRS; Oxoid) agar incubated at 30°C for 48 h in anaerobic condition. Fifteen colonies from MRS at each sampling point were randomly isolated and purified. The pH was measured by pH probe of a digital pH meter (micropH2001; Crison, Barcelona, Spain) according to the manufacturer's instructions.

### 3.2.7. Molecular typing by Rep-PCR LAB population

LAB isolates were subjected to DNA extraction and genetic fingerprints were obtained by using repetitive extragenic palindromic PCR (Rep-PCR) with the $(GTG)_5$ primer according to Iacumin *et al*. (2006b). The Rep-PCR profiles were normalized, and cluster analysis was performed using the BioNumerics software (version 6.1; Applied Maths, Sint-Martens-Latem, Belgium). The dendrograms were calculated on the basis of the Dice coefficient of similarity, with the unweighted pair group method using average linkages (UPGMA) clustering algorithm. After cluster analysis, 2 isolates from each cluster at >80% of similarity were selected and subjected to identification. The identification of LAB was performed by amplifying the 16S rRNA gene (Ercolini *et al*., 2010). Amplicons were sent for sequencing to GATC-Biotech (Cologne, Germany). To determine the closest known relatives of the 16S rRNA gene sequences obtained, searches were performed in public data libraries (GenBank) with the BLAST search program.

### 3.2.8. Statistical analyses

For taxonomic composition alpha diversity indices were calculated using the diversity function of the vegan package (Dixon, 2003). Indices were analysed using the pairwise comparisons using Wilcoxon rank sum test to assess differences between the batches. The OTU table obtained by metaphlan2 was used to produce the Principal component analysis (PCA) in R environment (www.r-project.org) by using the made4 package of R. ADONIS and ANOSIM statistical test was used to detect significant differences in the overall microbial community by using the OTU table. Not-normally distributed data were evaluated by Wilcoxon matched pairs test or the Kruskal–Wallis test as appropriate.

The normalized KEGG gene table was used to obtained the Co-Abundance Gene Groups (CAGs) by using the canopy-based algorithm. KEGG genes were clustered into CAGs and their abundance was calculated as the sample-wise median gene abundance (Nielsen *et al*., 2014; Zhang *et al*., 2015).

Determination of differentially abundant KEGG genes was conducted using the DESeq2 package (Love *et al*., 2014) in R as well as the GAGE Bioconductor package (Luo *et al*., 2009) in order to identify genes and biological pathways overrepresented or underrepresented between samples. P values were adjusted for multiple testing using the Benjamini-Hochberg procedure, which assesses the false-discovery rate (FDR).
Pairwise Spearman's correlations between taxa, CAGs, and volatile organic compounds were assessed by the R package psych, and the significant correlations (FDR, <0.05) were plotted in a correlative network by using Cytoscape v. 2.8.143. All the results are reported as mean values of 3 replicates for sampling point.
The genes presence/absence table obtained from the reconstructed bins was used to calculate the distance matrix on Bray Curtis's distance by the *vegdist* function in package vegan of R. The matrix was used to build the principal coordinate analysis (PCoA) through the *dudi.pco* function in made4 package. The website iTOL v5 was used to visualize the phylogenetic trees of the pangenome obtained through Roary.
One-way ANOVA was used to analyze the effect of ripening time on the dependent variables (pH, microbial count and volatilome data) separately for each batch. This statistical analysis was carried out by the software IBM SPSS® Statistics 25 using the Duncan's Multiple Range Test (MRT) post hoc test and 0.05 as level of significance.

### 3.3. Results

#### 3.3.1. Microbiota composition of sausages

The microbiota composition through fermentation obtained by metaphlan2 showed the dominance of *L. sakei* (around 40% of the relative abundance in all samples, Figure 1), followed by *L. curvatus* (15%) and *P. pentosaceous* (15%). We also identified minor populations composed by *Leuconostoc carnosum, Levilactobacillus brevis* and *Lactiplantibacillus plantarum.* By comparing the three batches we can clearly observe a specific minor microbiota that drove the cluster separation (Supplementary Figure 1). In detail samples manufactured in February were characterized by the presence of *L. carnosum, Leuconostoc mesenteroides* and *P. pentosaceus*, while *Lev. brevis* and *L. curvatus* were associated with samples manufactured in March (Figure 1). The May production was characterized by *L. sakei*, *Lactococcus lactis* and *Leuconostoc citreum.*
Moreover, the alpha diversity indexes didn't show differences in terms of observed species and chao1 index, but only the Shannon index showed differences (FDR < 0.05) between samples belonging to the February batch across time and between the three batches (Supplementary Figure 2).

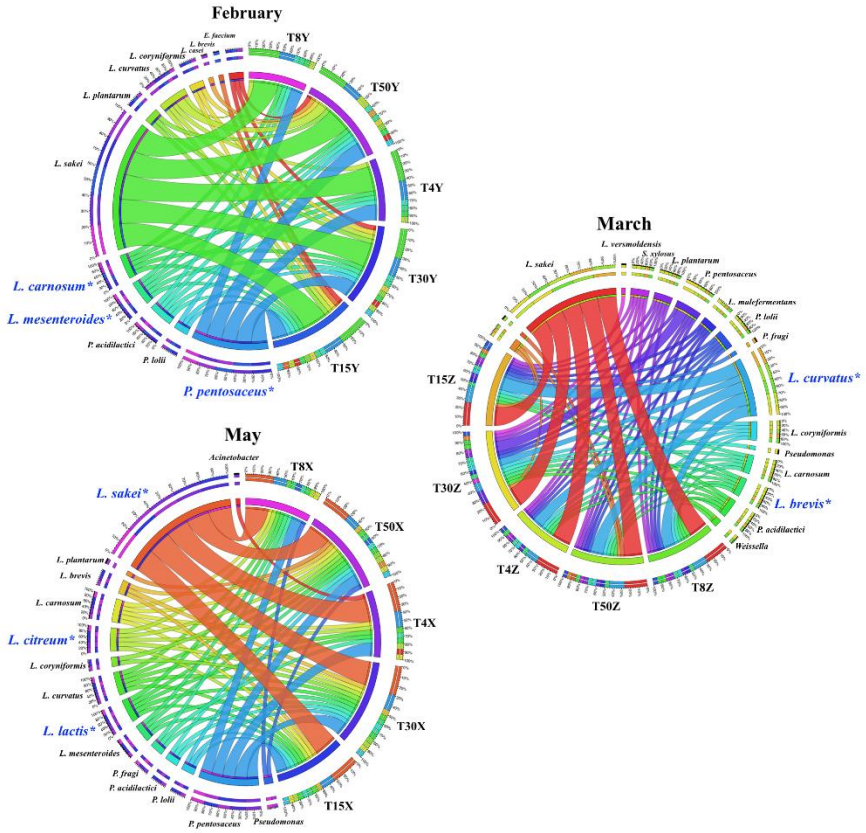


Figure 1. Circular ideogram showing the microbiota distribution among sausages from February, March and May batch. OTUs and samples are connected with a ribbon, and its thickness is proportional to the abundance of an OTU in the connected sample. The outer circle displays the proportion of each OTU in a given sample and vice versa. Taxa highlighted in blue denote OTU abundance significantly present in a given batch (FDR < 0.05, pairwise comparisons using Wilcoxon rank sum test).

### 3.3.2. Functional diversity of sausages

A total of 126.54 Gbp of raw reads were generated and after host (*Sus Scrofa*) sequence removal and quality filtering 35.34 Gbp of clean reads were used for further analysis. For each sample approximately 2.35 Gbp of clean reads were obtained (data not shown). A *de novo*-performed assembly generated a total of 1029546 contigs of more than 1000 bp in length, with an average N50 of 1090.4 bp (data not shown). The KEGG analysis assigned 2067 genes to 21 pathways. The pathway enrichment analysis performed through GAGE showed that March had lower KEGG pathways related to the biosynthesis of amino acids, valine, leucine and isoleucine biosynthesis, cysteine and methionine, amino sugar and nucleotide sugar, alanine, aspartate and glutamate metabolism, one carbon pool by folate and quorum sensing related genes, if compared against February and May. Moreover, by comparing May versus February, May displayed higher KEGG genes related to butanoate, methane, sulphur and pyruvate metabolism (data not shown).

### 3.3.3. Different distribution of genes repertoire according to season

Due to the observed different association between microbes and batches, a diverse distribution of genes repertoire according to season was noted, as well. For carbohydrate metabolism February (especially at the end of fermentation) showed higher KEGG gene count (FDR <0.05) of alcohol dehydrogenase (EC:1.1.1.1)

responsible for ethanol production from acetaldehyde, butanediol dehydrogenase/diacetyl reductase (EC:1.1.1.4; EC:1.1.1.303) involved in the production of acetoin, and D-lactate/ L-lactate dehydrogenase for the production of lactate from pyruvate (EC:1.1.1.28; EC:1.1.1.27) (data not shown).

The February batch also showed highest (FDR<0.05) counts of shikimate dehydrogenase (EC:1.1.1.25) involved in the biosynthesis of folates and aromatic amino acids (phenylalanine, tyrosine, tryptophan and indole) and tryptophan synthase (EC:4.2.1.20) involved in the interconversion between serine and tryptophan and between tryptophan and indole (data not shown) derived from the amino acids metabolism. Branched−chain amino acid aminotransferase (EC:2.6.1.42), as well as ketol−acid reductoisomerase (EC:1.1.1.86) coming from amino acids metabolism and catalyzing the conversion from valine to methyl-oxo butanoate were found most abundant in February.

The March batch displayed the highest counts of KEGG genes for carbohydrate metabolism like acetate kinase (EC:2.7.2.1), aspartate-semialdehyde dehydrogenase (EC:1.2.1.11) and acetyl-CoA C-acetyltransferase (EC:2.3.1.9) playing an important role in the production of propanoate and butanoate (FDR <0.05, data not shown). In addition, malate dehydrogenase (EC:1.1.1.38) that boosted the conversion of pyruvate to malate and several KEGG genes involved in the hydrolyzation of oligosaccharides as alpha−glucosidase (EC:3.2.1.20), alpha−N−arabinofuranosidase (EC 3.2.1.55) were associated with March samples (data not shown).

March samples were also rich in KEGG genes of the amino acids metabolism related to biogenic amine biosynthesis like agmatine deiminase (EC:3.5.3.12), thus taking part in the putrescine synthase pathway, converting agmatine into N-carbamoyl-putrescine, a precursor of putrescine. In addition, KEGG genes responsible for arginine interconversion, such as arginine deiminase (EC:3.5.3.6) (data not shown), were associated with March. Genes belonging to glycerophospholipid metabolism, such as glycerol-3-phosphate dehydrogenase (NAD(P)+) (EC:1.1.1.94) and glycerol-3-phosphate cytidylyltransferase (EC:2.7.7.39) (data not shown), were more abundant in March samples.

May samples displayed at the end of fermentation the highest abundance of methylglyoxal synthase (EC:4.2.3.3) and glutamate decarboxylase (EC:4.1.1.15) that from alanine route convert L-glutamate in 4-aminobutanoato and ribokinase (EC:2.7.1.15) in the pentose phosphate pathway (data not shown).

May samples were also rich in threonine synthase (EC:4.2.3.1) and in genes of histidine metabolism as histidinol dehydrogenase (EC:1.1.1.23), linked to the pentose phosphate pathway (data not shown). In addition, we observed the highest presence of 1,3−propanediol dehydrogenase (EC:1.1.1.202), a key KEGG gene involved in the lipid metabolism that allows the interconversion of propanal to propanol (data not shown).

### 3.3.4. Co-Abundance Gene Groups (CAGs)

We established co-abundance associations of KEGG genes and then clustered into sixteen co-abundance groups (CAGs) in order to identify signature patterns in the different batches (Supplementary Figure 3). CAG2 and CAG3 were associated with the February batch and CAG6 with the March batch (FDR < 0.05). In details CAG6 was composed by KEGG genes related to pyruvate metabolism and arginine and proline metabolism, in particular agmatine deiminase (EC:3.5.3.12), involved in the biogenic amines production. CAG2 and CAG3 were related to sulphur metabolism, phenylalanine, tyrosine and tryptophan biosynthesis, methane metabolism, glycolysis, butanoate metabolism, pentose phosphate pathway and fatty acid biosynthesis.

### 3.3.5. Strain-Level Differences association - Draft genomes by metagenome binning

Several bins were identified in the sausage metagenomes. We found the presence of 6, 7 and 9 *L. sakei* draft genomes in the batches of February, March and May respectively; 5 and 2 *L. curvatus* in the batches of March and February, respectively; 6, 2 and 5 *P. pentosaceus* in the batches of February, March and May, respectively (data not shown). The genomes of *P. pentosaceus* were reconstructed mainly from the February batch, while genomes of *L. curvatus* and *L. sakei* from the batches in March and May, respectively (data not shown). Following Bowers *et al* (2017) only 2 strains of *L. sakei* were classified as high quality, 4 medium quality and the other one low quality; for *L. curvatus* 6 were medium quality and one low quality; *P. pentosaceus* were classified as 2 high quality, 5 medium quality and 6 low quality. Using the metagenomes, we reconstructed the phylogenetic tree for the three bacterial species, but we didn't obtain a clear separation based on the batch (Figure 2).

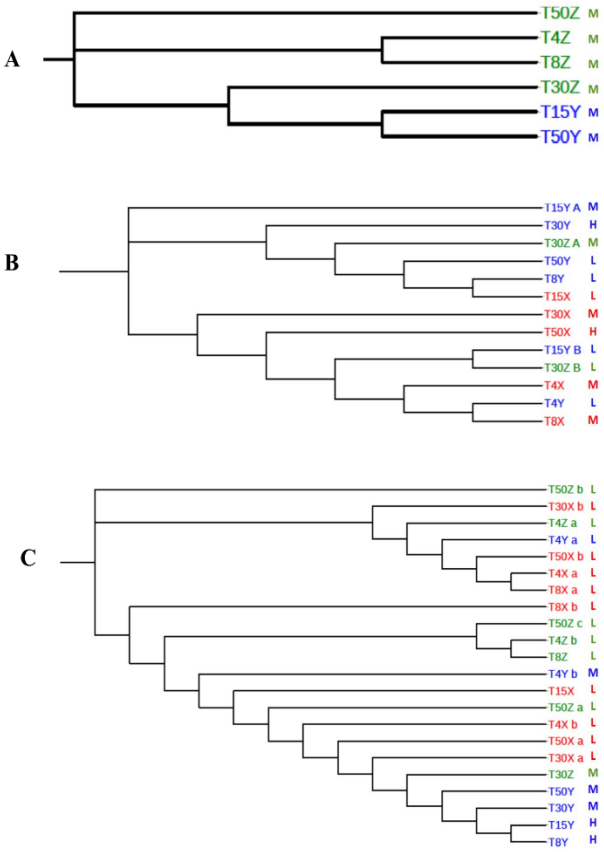


Figure 2. Single-Nucleotide Polymorphisms phylogenetic tree built on concatenated *Latilactobacillus curvatus* (A); *Pediococcus pentosaceus* (B) or *Latilactobacillus sakei* (C) genes extracted from assembled metagenomes. The quality of the reconstructed genome is also indicate: low (L; <50% complete, <10% contamination), medium (M; >50% complete, <10% contamination), high (H; >90% complete, <5% contamination). The different colors correspond to the different batches: February in blue, March in green and May in red.

By a comparative analysis between the three groups of reconstructed genomes *L. sakei*, *L. curvatus* and *P. pentosaceus* (Figure 3) we were able to observe specific genes associated with each group of strains. We found that D-lactate dehydrogenase genes were present only in *P. pentosaceus* genomes and enolase and mevalonate kinase genes showed the highest prevalence for this species. *L. sakei* genomes displayed the highest presence of 3-dehydroquinate dehydratase (belonging to the biosynthesis of secondary metabolites pathways), arginine deiminase and carbamate kinase 1 (belonging to the arginine biosynthesis pathways), lactose permease and L-lactate dehydrogenase genes. Interesting phosphate propanoyl transferase and propanediol dehydratase genes (belong to propanoate metabolism pathways) were found in *L. curvatus* and *P. pentosaceus* genomes, but were absent in *L. sakei* genomes. Acetolactate synthase and adenine deaminase genes showed the lowest presence in *L. sakei* genomes.

*L. sakei* and *P. pentosaceus* genomes were associated with key genes of glycolysis and pyruvate metabolism (6-phosphofructokinase, pyruvate kinase, pyruvate oxidase) and pentose phosphate pathway (glucose-6-phosphate 1-dehydrogenase, ribokinase). The presence of these genes was low in *L. curvatus* genomes.

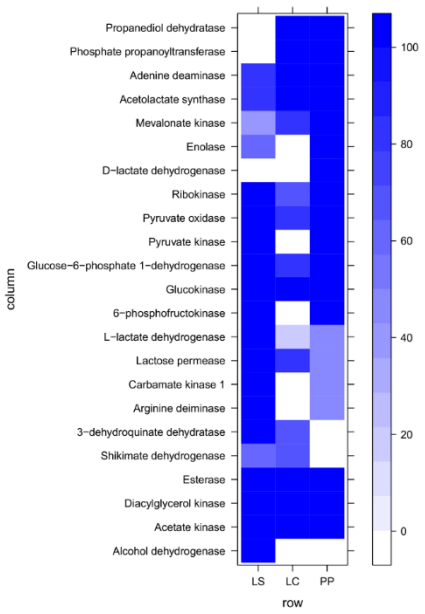


Figure 3. Occurrence (%) of genes involved in metabolic pathway genes in *Latilactobacillus sakei* (LS), *Latilactobacillus curvatus* (LC), *Pediococcus pentosaceus* (PP) reconstruct genomes.

### 3.3.6. *L. sakei* strain-level differences

It is well known that fermented sausages are an ecological niche for *L. sakei* and in order to discover its potentials during fermentation we further analysed in depth its genome content. For this purpose, to confirm the results obtained by the binning methods after metagenome assembly, contigs belonging to *L. sakei* 23K (1.99 Mbp in size), used as reference strain, were extracted. Reconstructed genomes from contigs of each sample displayed the presence of a part of the full genome (due to assembly and coverage limitations) of about 1.33 Mbp in median value (data not shown). Principal coordinates analysis (PCoA) (Supplementary Figure 4) based on shell and cloud genes of the 15 reconstructed *L. sakei* clearly highlights a separation of the strains based on the production batch.

Based on occurrence of *L. sakei* gene repertoire we observed that genomes reconstructed from March had several genes with higher prevalence, if compared with February and May (Figure 4). In detail, genes involved in glutamate metabolism and pentose phosphate pathway like pyruvate transaminase and ribokinase were dominant in this batch. Moreover, the lowest presence of agmatine deiminase and putrescine carbamoyl-transferase gene (genes involved in the agmatine deiminase pathway) was determined. Other genes not observed in May-associated *L. sakei* genome ($P < 0.05$) were: carbamate kinase gene (involved in purine metabolism, glutamate metabolism, arginine and proline metabolism and nitrogen metabolism), ribokinase and lactose permease.

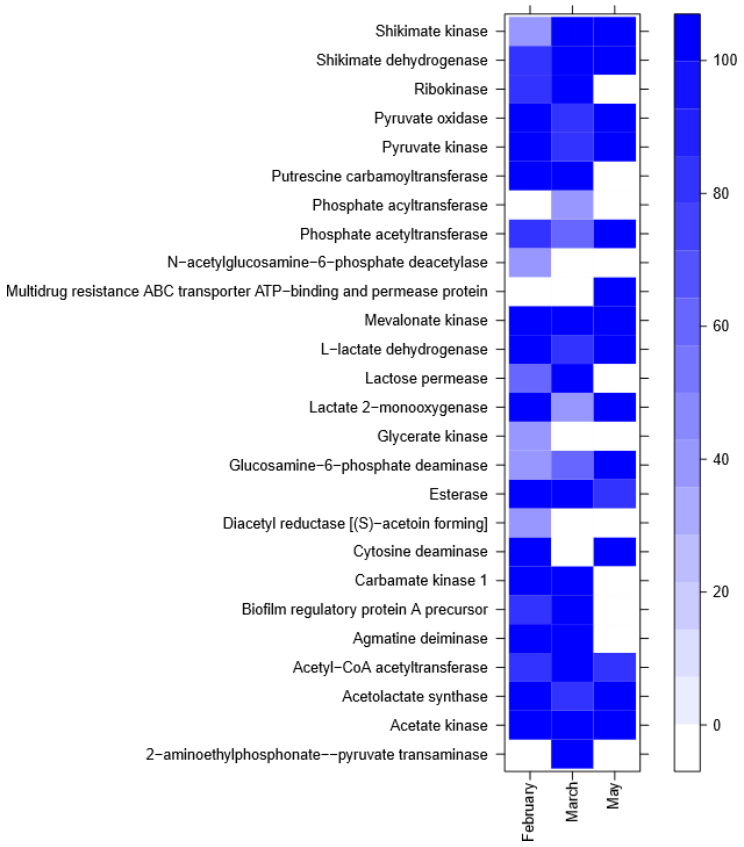


Figure 4. Occurrence (%) of genes involved in metabolic pathway genes in reconstructed genomes of *Latilactobacillus sakei* between the three batches.

### 3.3.7. Volatilome profile of fermented sausages

A specific signature of the volatilome characterized each batch (Supplementary Table 1). In detail the March batch showed the highest values of 2-butanone, methyl propionate, ethyl propanoate, 2-butanol, 2,3-pentanedione, hexanal, methyl hexanoate, 2,3-octanedione, propanoic acid, 2-octen-1-ol and hexanoic acid ($P < 0.05$, Supplementary Figure 5). In addition, the three batches at the end of ripening were different ($P < 0.05$) for the concentration of the following compounds: isobutyric acid showed the lowest values in February samples, ethyl isovalerate had the lowest values in samples from March, and octanal, ethyl lactate and butanoic acid had the lowest values in May samples (Supplementary Figure 5).

### 3.3.8. Correlations between microbiota, VOCs and CAGs

Our understanding of sausage microbiome is a strict connection between microbes' function and metabolomic development. In our study we observed that February samples at end of ripening showed high level of ethanol and ethyl lactate and the presence of these VOCs were linked to the high abundance of the KEGG genes of alcohol dehydrogenase (EC:1.1.1.1) and D-lactate dehydrogenase (EC:1.1.1.27) (Figure 5A) belonging to CAG2 and CAG3 groups respectively. The genome analysis of *P. pentosaceus* confirmed the specific presence of the D-lactate genes (Figure 3).

In addition, we observed that the March batch showed high levels of butanoic acid, methyl propionate, propanoic acid and ethyl propanoate (Supplementary Figure 5). The presence of these VOCs was confirmed by the high abundance of the KEGG genes belonging to butanoate and propanoate pathways (CAG6 group) (Figure 5B). Genes belonging to propanoate pathways were found also in *L. curvatus* genomes.

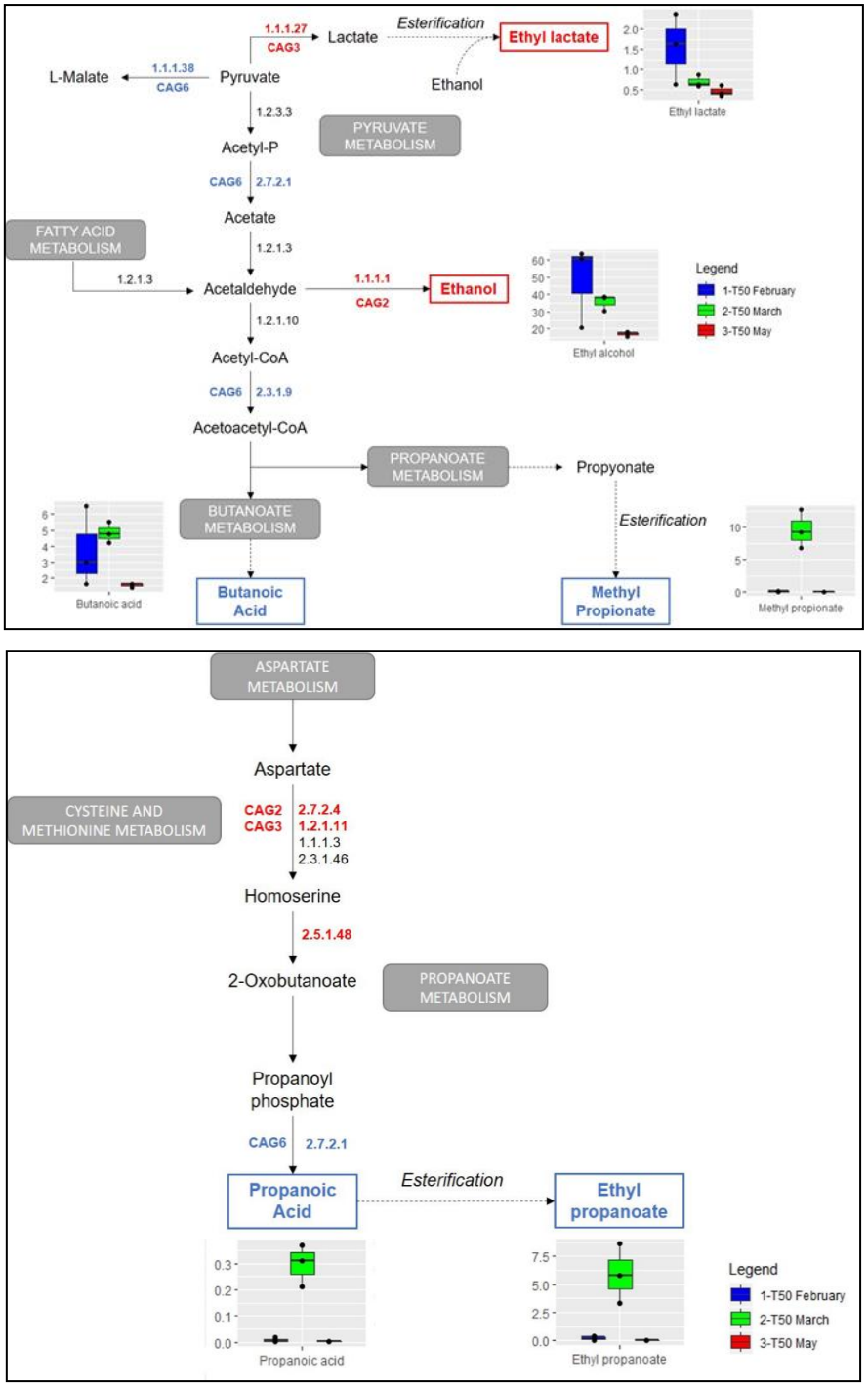


Figure 5. Biosynthesis of volatile compounds from pyruvate (panel A) and aspartate (panel B). Only KEGG genes identified in the samples analyzed are reported. The graph showed volatile compound after 50 day of fermentation and KEGG gene (and CAG) associated with February production highlighted in red and March production highlighted in blue.

In order to have a better comprehension of this fermented food model, a correlation network (Figure 6) based on microbiota, VOCs and CAGs was visualized. Through the network it was determined that the presence of specific species was positively or negatively correlated to specific VOCs and CAGs. In fact, focusing only on the three species that characterized each batch, we could observe that *P. pentosaceus* was positively correlated with hexanal, heptanone, acetoin, ethyl alcohol, heptanal, nonanal, octanal as well as with CAG2, CAG3 and CAG5 (Figure 6, FDR<0.05). *L. sakei* was positively correlated to ethyl isovalerate and negatively correlated to propanoic acid, acetic acid and dimethyl sulfone. *L. curvatus* showed a positive correlation with CAG6 and a negative correlation to heptanal, ethyl isovalerate and CAG5 (Figure 6, FDR<0.05).

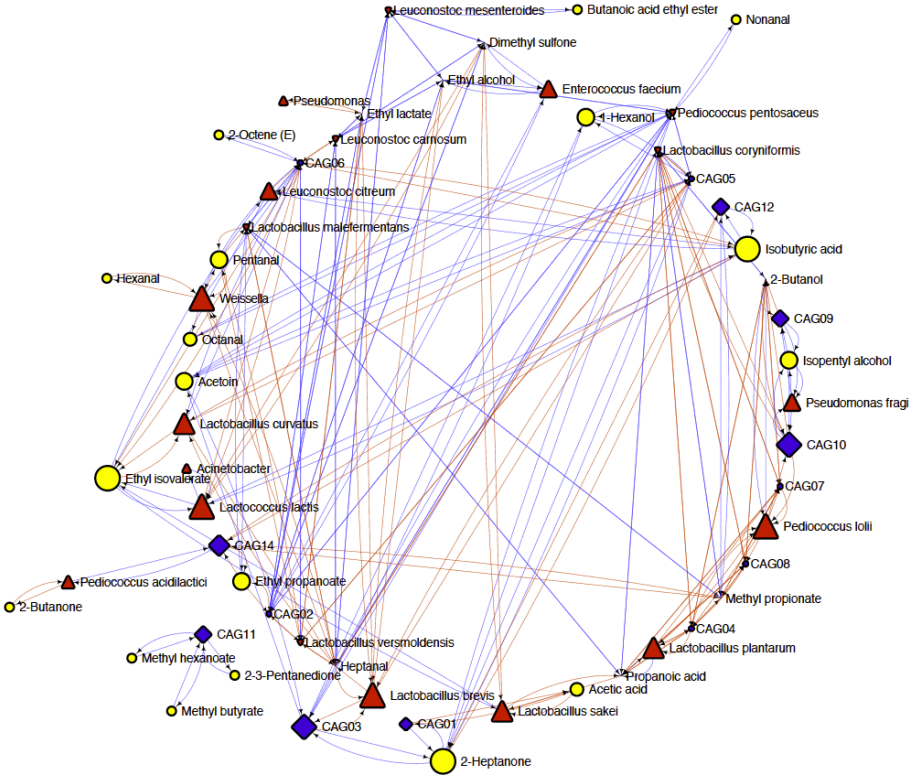


Figure 6. Correlation between volatilome data (yellow circle), taxa (orange triangle) and CAGs (blue rhombus). Correlation network showing significant (false-discovery rate FDR <0.05) Spearman's correlations between KEGG genes, VOCs, and taxa. Node sizes are proportional to the numbers of significant correlations. Colors of the edges indicate positive (blue) or negative (red) correlations.

### 3.3.9. Microbiota evolution and culture dependent strain diversity of LAB population

During ripening (Supplementary Table 2) pH decreased across time in all batches from around 6 at time zero (February: 6.49 ± 0.10; March: 6.03 ± 0.09; May: 5.99 ± 0.04) to about 5 (February: 5.29 ± 0.03; March: 5.72 ± 0.18; May 5.35 ± 0.1) at the end of the study, with the March batch maintaining a higher pH if compared with the others (Supplementary Table 2, P<0.05). Regarding the LAB population, by culture dependent approach, we observed a load increase from around 3 to 9 Log CFU/g in the first day of ripening with no further increase till the end, with the highest values in May samples (Supplementary Table 2, P<0.05).

By culture dependent approach we isolated 224 strains from MRS plates. The results of the identification of the colonies isolated from the three batches showed that: February was characterized by 77% *L. sakei* and 23% *P. pentosaceus;* March samples showed 62% *L. sakei* and 32% *L. curvatus*; and May samples displayed 97% of *L. sakei* and 3% of *P. pentosaceus* (data not show). It should be pointed out that *L. curvatus* was never isolated from May and February while *P. pentosaceus* was never isolated from March samples. The molecular characterization performed by REP fingerprinting approach on the main population isolated from all batches belonging to *L. sakei* showed the presence of 51 REP-biotypes. In detail it was possible to observe that March samples showed the highest presence of unique *L. sakei*, while May and February had several common *L. sakei* REP-biotypes (data not show). This trend was also observed for the pH, in fact the batch from March showed different values at the last two sampling times (Supplementary Table 2, $P > 0.05$).

### 3.4. Discussion

We characterized the metagenomes of spontaneous fermented sausages in order to provide evidence that the presence of different strains can affect specific genomic repertoires and affect the organoleptic characteristics of the final product. The differences shown in the three batches at the end of the ripening where due to the different metagenome content deriving from the initial microbial composition and its development. The metagenome reconstruction showed that the highest number of *L. sakei* strains were extracted from the metagenome of the batch from May, *P. pentosaceus* from the one from February and *L. curvatus* from March samples. Data from culture dependent methods confirmed this observation.

February samples were rich in *P. pentosaceus* strains and showed its high acidification capability of quickly lowering the pH, as already reported by Aro *et al*. (2010). This capability could be a positive survival strategy in fermented food and a positive quality for the use of this species as a starter culture in fermented meat for consumers that prefer high acidity products (Kingcha *et al*., 2012; Nur and Aslim, 2010; Porto *et al*., 2017). February samples showed also the highest value for specific volatile compounds related to herbs and floral note (e.g. octanal) (Olivares *et al*., 2009). Aldehydes are considered fresh and agreeable at low levels, but unpleasant and rancid when concentrations rise; alcohols and esters are essential in order to obtain the proper fermented sausage aroma by adding fruity and sweet notes to the aroma (Casaburi *et al*., 2015; Stahnke, 1994). We observed in February samples the highest level of alcohol dehydrogenase (EC:1.1.1.1) and D-lactate dehydrogenase (EC:1.1.1.27) that boosted the production of volatile compounds like ethanol and ethyl lactate. *P. pentosaceus* is responsible for this behaviour due to the presence of the D-lactate dehydrogenase gene. The importance of D-lactate dehydrogenase gene in *Pediococcus* sp. is related to the fact that is a key gene involved in the production of 3-Phenyllactic acid (PLA), a novel antimicrobic molecule used to extend the shelf life of food (Mu *et al*., 2012; Yu *et al*., 2014). February samples also showed the highest counts of shikimate dehydrogenase (EC:1.1.1.25) belonging to the shikimate pathway. The shikimate is an intermediate in the chorismate pathway, which serves as a branching point toward the biosynthesis of aromatic amino acids and pABA (Gupta *et al*., 2017). Since *P. pentosaceus* strains were more present in the samples from the February

batch, we could assume that the high presence of these volatile compounds in February samples was linked to the metabolic activity of this species. Some studies report that the inoculation of *P. pentosaceus* could prevent an excessive lipid oxidation that can be the cause of quality deterioration in meat products (off-flavour). In addition *P. pentosaceus* strains could also promote the formation of alcohols originating from the amino acid catabolism, for example the formation of ethanol from the reduction of acetaldehyde in the presence of alcohol dehydrogenase (Chen *et al*., 2015a, 2015b). In fact, Figure 5A shows acetaldehyde production and consequently ethanol production from fatty acid metabolism.

*L. curvatus* was observed as a key species in March samples, where genes related to agmatine deiminase (EC:3.5.3.12) confer acid resistance in LAB species (Ammor and Mayo, 2007; Lucas *et al*., 2007). Regarding the metabolome profile, we observed that methyl propionate, butanoic acid, propanoic acid and ethyl propanoate were abundant due to the highest presence of KEGG genes related to propanoate and butanoate metabolism. These specific metabolites confer to the sausages apricot taste (2-butanone), butter and cheese flavour (ethyl propionate, ethyl propanoate, 2-butanol, 2,3-pentanedione) (Olivares et al., 2009) and grass, fatty and fruity sweet odour (hexanal) (Casaburi *et al*., 2015). The dry sausage aroma is often associated with the dominance of 2-methyl ketones, whereas a rancid aroma is associated with high concentration of hexanal (Montel *et al*., 1998). Diacetyl, acetic acid and hexanal are also associated with buttery, vinegar and green odour notes, respectively (Rimaux *et al*., 2012). Samples from the batch of March were considered unacceptable from a sensorial point of view, due to the presence of hexanal almost three times higher compared with the other batches. It was already reported that a strong presence of *L. curvatus* could be the cause of an unusual smell (off-flavours) linked to the perception of 'too strong' (Visessanguan et al., 2006). Visessanguan *et al* (2006) reported that the off-flavours observed in sausages inoculated with high concentrations of *L. curvatus* were due to a higher presence of free fatty acids connected with a strong lipid oxidation. On the other hand, it is not easy to link the higher presence of hexanal and of the other VOCs found in March samples only to the *L. curvatus* activity. Some works underline a similar metabolic activity between *L. curvatus* and *L. sakei* strains (Chen *et al*., 2017; Freiding *et al*., 2011; Tabanelli *et al*., 2012) and consequently the similar development in metabolites. On the contrary, in our study the genes belonging to CAG6 group (genes belonging to butanoate and propanoate pathways) were associated to *L. curvatus*, which was isolated only in March. From genome reconstruction we observed the presence of genes related to the propanoate metabolism associated with *L. curvatus* and we can speculate its ability to push the propanoic and butanoic route with the consequential formation of the relative VOCs that we found in high concentration in the March batch especially at the end of ripening.

*L. sakei* was positively correlated to ethyl isovalerate production, especially in May samples. In fact, in May the highest presence of isobutyric acid and ethyl isovalerate was determined. Isobutyric acids can originate from valine, leucine and isoleucine and their description is related to sweet, sickly and malty odour (Dainty *et al*., 1985). *L. sakei* strains belonging to the May batch showed the lowest occurrence of acetate kinase gene (a key gene in the glycolysis pathway), carbamate kinase (involved in purine, glutamate, arginine and proline and nitrogen metabolism), lactose permease, putrescine carbamoyl-transferase (involved in the agmatine deiminase

pathway) and ribokinase (involved in the pentose phosphate pathway). Since all these genes belong to the main route for the production of aromatic VOCs, we can expect less aromatic flavours in the product obtained in May. A confirmation of this hypothesis came from the volatilome profile at the end of fermentation where the presence of 2-butanone, methyl propionate, ethyl alcohol, ethyl propanoate, 2-butanol, propanoic acid, butanoic acid and hexanoic acid were lowest.

Our pangenomic analysis highlighted the presence of specific fermentation-driven strain-level profiles of *L. sakei*. The reconstructed genomes from March samples had the higher prevalence of genes like ribokinase and key genes for the glycolysis and pentose phosphate metabolism. On the contrary *L. sakei*-genomes from May showed a lower prevalence of these specific genes and a consequently low presence of volatile compounds interesting for the final organoleptic properties.

Our analysis revealed that the majority of the completely reconstructed strains belonged to *L. sakei* and that most of the key enzymes related to volatile compounds metabolism were present in *L. sakei* genomes. This confirms that this species dominates the microbiota of fermented meats and has a fundamental role for the characterization of the organoleptic profile. The genomic intra and inter-species diversity at strains level influence the final quality of the products, as well as the development of other indigenous microbiota.

### 3.5. References

References of this chapter are integrated into the overall reference section (Chapter 7).

### 3.6. Data availability

Sequences have been uploaded to the National Center for Biotechnology Information Sequence Read Archive (Bioproject ID PRJNA636619).

# Chapter 4

## Mycobiota dynamics and mycotoxin detection in PGI Salame Piemonte

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Submitted

**Abstract**

Aims: The complex mycobiota that colonizes traditional fermented sausages plays an important role in the organoleptic properties of such products. The aim of the present study was to investigate fungal diversity and dynamics during maturation of PGI *Salame Piemonte*.

Methods and Results: Casing and meat samples were collected at five sampling times from three different batches produced in the same factory and analysed using culture-dependent and -independent approaches. *Penicillium nalgiovense*, which was deliberately inoculated, and *Debaryomyces hansenii*, were the most dominant taxa in casings. Several other fungi mainly belonging to *Penicillium crustosum*, *P. glabrum*, *P. nordicum*, *Cladosporium* spp., *Candida sake*, *C. zeylanoides* and *Yarrowia divulgata* were also identified. The casing mycobiota was compared to that of the meat using a metataxonomic approach and a higher fungal diversity was observed in meat as compared to casings. Mycotoxins and penicillin G were monitored using QTOF LC-MS and only trace amounts of roquefortine C were detected in two batches.

Conclusions: The present study highlighted the diversity of Salame Piemonte mycobiota and the important contribution of autochthonous fungi to its diversity. The absence of mycotoxins and penicillin G confirmed the high hygienic quality of the studied product regarding fungal and mycotoxin contamination.

Significance and Impact of Study: For the first time, this study provides insights about the Salame Piemonte mycobiota which together with the bacterial microbiota and Salame Piemonte process specifications are responsible for the product organoleptic properties.

### 4.1. Introduction

Fermented meat products constitute a significant part of the Mediterranean diet and their production and commercialization contribute to the local economies (Baka *et al*., 2011). In the last decades, many studies have focused on the microbiota of fermented sausages (Ferrocino *et al*., 2018). However, most of the studies only focused on bacterial microbiota, *i.e*., lactic acid bacteria (LAB) and coagulase-negative staphylococci (CNS) that are the most important bacterial groups involved in the fermentation process (Franciosa *et al*., 2018). To date, fungal diversity and their functional roles in these products have been less studied (Toldrá *et al*., 2014).

Many fungal species are well adapted to the ecological conditions encountered during meat fermentation including intrinsic (pH, water activity, NaCl content, natural and intentionally added antimicrobials, available nutrients) and extrinsic factors (temperature, humidity) (Sonjak *et al*., 2011). That is why many fermented meat products, including fermented sausages, are characterized by fungal growth (both yeasts and molds) on the casing surface. Their metabolic activities, including glucidolytic, proteolytic and lipolytic activities, can be linked to aroma compound production that contribute to the typical cured flavours of the final products (Cruxen *et al*., 2019; Murgia *et al*., 2019; Parussolo *et al*., 2019a; Vila *et al*., 2019). Other functional roles of desirable fungi include competition/exclusion of undesirable fungi (including mycotoxin producers) and surface colonization which changes the visual aspect of the products (Pitt and Hocking 2009). This latter role also helps to prevent excessive drying, protects against oxidative processes through peroxide metabolization and $O_2$ consumption, improves meat colour, increases pH via lactate utilization and deamination activities and facilitates sausage casing peeling through hyphae penetration into the casing (Belleggia *et al*., 2020b; Comi and Iacumin, 2013; Toldrá *et al*., 2014).

Given the important functional roles played by this mycobiota, manufacturers often use selected fungal strains, especially the mold species *Penicillium nalgiovense* (Sunesen and Stahnke 2003) and the yeast species *Debaryomyces hansenii* (Murgia *et al*., 2019). These species are inoculated onto the casing surface to better control the fermentation and ripening process and improve overall product quality by ensuring more constant quality attributes (Meftah *et al*., 2018). The latter species are also frequently encountered in spontaneously fermented sausages (Montanha *et al*., 2018; Vila *et al*., 2019), but many other indigenous fungal species, including desirable and undesirables ones, can also be found in these products. Given the positive impact fungal species can have on product quality, it is of interest to investigate fungal diversity to improve our knowledge on this kind of product.

As mentioned above, growth of undesirable mold species producing mycotoxins, i.e., secondary metabolites toxic to human health, may also occur during the fermentation process (Meftah *et al*., 2018; Pleadin *et al*., 2017), especially in conditions where air quality control, among other factors, is unsatisfactory. The main mycotoxin producers encountered in fermented meats belong to *Aspergillus* and *Penicillium* spp. (Montanha *et al*., 2018). More specifically, the most common species are *Penicillium crustosum* that can produce penitrem A and roquefortine C (Coton and Dantigny, 2019) and *Penicillium nordicum* and *Penicillium verrucosum* capable of producing ochratoxin A (OTA) (Iacumin *et al*., 2009; Samson *et al*., 2010). For the latter mycotoxin, the Italian Ministry of Health recommended, in meat or meat products, a maximum value of 1 µg/Kg of OTA

(Ministero della Sanità 1999). It is worth mentioning that there is currently no European regulation specifying maximum mycotoxin concentrations in meat products in contrast to other foods, e.g., cereals and cereal-based products, groundnuts, oilseeds, dairy products, for which maximum levels are set by the European Union in EU Regulation 1881/2006 (Official Journal of the European Union 2006; Montanha *et al*., 2018). In addition to mycotoxins, several *Penicillium* species belonging to the Chrysogena section, including *P. nalgiovense,* are able to produce the antibiotic penicillin (Papagianni *et al*., 2007; Parussolo *et al*., 2019a). Penicillin presence in food is undesirable as it can cause human allergies (Stone *et al*. 2020) and could also lead to resistance development in food-borne bacteria and/or commensal gut bacteria as well as the transfer of resistance genes to pathogenic bacteria (Verraes *et al*. 2013). Identifying fungal species that compose the fermented sausage casing mycobiota and quantifying potential hazardous secondary metabolites is therefore essential as part of safety risk assessment.

In this context, the aim of the present study was to investigate mycobiota diversity and dynamics of a typical fermented sausage, *Salame Piemonte,* granted with a Protected Geographical Indication (PGI) quality label and produced in the Italian Piedmont region, using culture-dependent and -independent approaches and determine mycotoxin and penicillin content using QToF LC-MS.

## 4.2. Material and methods

### 4.2.1. Sausage manufacture and sampling

Three independent productions were carried out in an Italian factory located in the geographical area of Piedmont region (North-West of Italy) following the Salami Piemonte PGI quality label specifications. The formulation used in the manufacturing included pork lean meat from the shoulder and fat from the belly, salt (maximum 3%), pepper (maximum 0.4%), spices and aromatic plants crushed or infused with wine. The batches were produced in February, March and May, 2018. Concerning sampling, a total of 45 spontaneously fermented sausages and 45 natural casing samples, from the three batches, were collected at 4, 8, 15, 30 and 50 maturation days. At each sampling time, pH was measured from the first ten-fold serial dilution, and aw measurements directly from the meat sample using a digital pH meter (micropH2001; Crison, Barcelona, Spain) and a calibrated electric hygrometer (HygroLab; Rotronic, Bassersdorf, Switzerland) according to the manufacturer's instructions, respectively.

PGI specifications for this product permit the use of commercial starter cultures including bacteria and fungi for meat and casing inoculation. However, in the framework of this study, the salamis were only inoculated on the casing surface with a commercial starter culture of *P. nalgiovense.*

### 4.2.2. Culture-dependent analyses of meat and casing samples to assess fungal diversity

#### 4.2.2.1. Enumeration and isolation

From each sausage, 5 pieces (1 $cm^2$) of casing randomly removed under sterile conditions using a sterile scalpel or 10 g of meat were resuspended into 9 ml of sterile Ringer solution and vortexed vigorously for 1 minute.

Ten-fold serial dilutions were performed and 100 µL aliquots were plated in triplicate on malt extract agar (AMT, Oxoid) supplemented with 0.05 g $L^{-1}$ tetracycline (Sigma, Milan, Italy) (Greppi *et al*. 2015). Plates were incubated for 5 days at 25°C for fungal enumerations and isolations. Counts were expressed as mean values ($log_{10}$ colony-forming units CFU per $cm^2$ or g of sample) of three technical replicates ± standard deviation. Given the low abundances of fungi in meat (mean abundance $<10^3$ CFU $g^{-1}$) as compared to casing samples, culture-dependent identification was only performed on fungi collected from casing samples. Approximately 20 fungal isolates were randomly picked from each batch and sampling time and purified on AMT media. Then, yeast isolates were cryopreserved in 30% v v-1 glycerol at -80°C after growth in YPD broth (dextrose 2%, bacteriological peptone 1%, yeast extract 1%) for 24h at 25°C. For filamentous fungi isolates, mycelium plugs removed from agar plates were cryopreserved at -80°C in pure sterile glycerol. A total of 275 isolates from casing samples (including 132 molds and 143 yeasts) were collected and used for downstream analyses.

#### 4.2.2.2. Identification of fungal isolates

Moulds were presumably identified and dereplicated based on macroscopic and microscopic features (Pitt and Hocking 2009; Samson et al. 2010) and a total of 39 filamentous fungi were then selected for molecular identifications as described below.

Yeast isolates were dereplicated and presumptively identified by using the Fourier transform infrared (FT-IR) spectroscopy using a high-throughput system comprising a spectrometer (Tensor 27, Bruker Optics, Champs sur Marne, France) coupled to a high-throughput module (HTS-XT, Bruker Optics). After cultivation on yeast extract glucose chloramphenicol agar medium (YGC, Merck 1.16000) for 24 h at 27 °C, a half loop suspension of cells was suspended in 100 µl of deionised sterile water. Then, 25 µl of this suspension were deposited onto a ZnSe carrier and dried for about 45 min at 40 °C. Spectra acquisition and processing were performed as previously described (Kümmerle *et al*., 1998) and spectra were compared to the Technical University of Munich reference database comprising about 2500 FTIR reference spectra. Analyses were performed using three technical triplicates. Yeasts from each batch were grouped according to their spectrum using the OPUS software program (Bruker, France) and representative isolates from each cluster were selected for molecular identification (total of 40 isolates).

DNA was extracted from scraped colonies for yeasts or mycelial plugs for molds using the FastDNA SPIN Kit (MP Biomedicals, Illkirch, France) according to the manufacturer's instructions. DNA was then diluted to 50–100 ng µl-1 and kept at −20 °C until further analysis. For yeasts, the D1/D2 domain of the 26S rRNA gene was PCR-amplified using primers NL1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL4 (5′-GTCCGTGTTTCAAGACGG-3′) (Kurtzman and Robnett 1997). For filamentous fungi, the partial β-tubulin gene (presumptive *Penicillium* spp.), partial elongation factor α (EFα) (presumptive *Cladosporium* spp.) or internal transcribed spacer (ITS) region (other genera) were amplified using primers Bt2a (5′-GGTAACCAAATCGGTGCTGCTTTC-3′) and Bt2b (5′-ACCCTCAGTGTAGTGACCCTTGGC-3′) (Glass and Donaldson, 1995), EF1-728F (5′-CATCGAGAAGTTCGAGAAGG-3′) and EF1-986R (5′-

TACTTGAAGGAACCCTTACC-3′) (Carbone and Kohn, 1999) and ITS4 (5′-CCTCCGCTTATTGATATGC-3′) and ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) (White *et al*., 1990), respectively. PCR amplifications were carried out in a FlexCycler thermocycler (Analityk Jena, Germany) and PCR amplicons were sequenced at the Eurofins sequencing platform (Nantes, France) using the same primer pair. After sequencing assembly into contigs using the DNA Baser software (Heracle software, Germany), sequences were compared with the GenBank database using the "Basic Local Alignment Search Tool" (BLAST) (https ://www.ncbi.nlm.nih.gov/BLAST) for taxonomic assignment.

#### 4.2.3. Culture-independent analyses of meat and casing fungal communities using metabarcoding

DNA was extracted from meat and casing samples obtained at each sampling point after centrifugation at 13,000 g for 10 min of 1 ml of the first ten-fold serial dilution in sterile Ringer solution . After removing the supernatant, total DNA was extracted from pellets using the RNeasy Power Microbiome KIT (Qiagen, Milan, Italy) following the manufacturer's instructions. One microliter of RNase (Illumina Inc. San Diego. CA) was added to digest RNA in the DNA samples, with an incubation of 1 h at 37°C. DNA was quantified using the QUBIT dsDNA Assay kit (Life Technologies, Milan, Italy) and DNA concentration standardized at 5 ng $\mu L^{-1}$. DNA was subjected to amplification of the D1 domain of the 26S rRNA gene using the same primers (LS2-MF 5'-GAGTCGAGTTGTTTGGGAAT-3' and NL4 5'-GGTCCGTGTTTCAAGACGG-3') and procedure as described previously (Mota-Gutierrez *et al*., 2019). Briefly PCR was carried out using a mixture prepared with 12.5µl of the 2X Kapa HiFi HotStart ReadyMix Taq (Roche, Milan, Italy), 1µM each primer, 2.5µl of DNA template, and PCR-grade water. PCR were subject to the following conditions: thirty cycles of 30 s of denaturation (95 °C), 30 s of primer annealing (55 °C), and 30 s of primer elongation (72 °C), followed by a final elongation step (72 °C) of 10 min. PCR templates were then purified and processed following the Illumina metagenomic pipeline. Sequencing was performed in a MiSeq instrument in a 2X250bp configuration. After sequencing, reads were analysed by using QIIME following the detailed pipeline described by Mota-Gutierrez *et al*. (2019). The 26S rRNA gene database from the same authors was used for taxonomic assignment and OTUs centroids (picked at 99% of similarity) were double checked on BLAST suite tools.

#### 4.2.4. Statistical data analysis

OTU tables were rarefied to the lowest number of sequence/sample for each dataset. R was used to calculate alpha diversity index using the vegan function. Principal component analysis (PCA) was also used for the cluster analysis according to OTU composition and categorical variables. A one-way ANOVA was used to analyze the effect of ripening time on dependent variables (mycobiota) separately for each batch by using the IBM SPSS® Statistics 25 software with Duncan's Multiple Range Test (MRT) post hoc test with a significance level set to 0.05. The *psyc* function in R was used for correlation analysis of OTU data from casing and meat samples by spearman's rank method and the results were visualized using the *corrplot* function in R.

#### 4.2.5. Extrolite extraction, detection and quantification in casing samples

Since mycotoxins mostly accumulate in salami casing (Parussolo et al. 2019b), only casing samples were analyzed. Casing samples (0.4 g) from sausages matured for 15, 30 and 50 days were analysed after liquid-solid extraction. The extrolite determinations were then carried out on these samples by Quadrupole Time-of-Flight liquid chromatography coupled to mass spectrometry (QTOF LC-MS; 6530 Agilent, France). The following 17 extrolites were targeted: patulin (PAT), penitrem A (PEN A), andrastin A (AND A), mycophenolic acid (MPA), citrinin (CIT), ochratoxin A (OTA), cyclopiazonic acid (CPA), aflatoxin B1 (AFLB1), (Iso)-fumigaclavine A (ISO-FUMI A), sterigmatocystin (STERIG), griseofulvin (GRISEO), meleagrin (MELEA), eremefortins A (EREM A) and B (EREM B), roquefortine C (ROQ C), citreoviridin (CITREO) and penicillin G (PEN G). Compound characteristics used for their detection and quantification by QTOF LC-MS are shown in Supplementary Table 1.

For extrolite extraction, casing samples were vortexed for 2 min in 6 mL acetonitrile-methanol-water (30/30/40, v/v/v) solution and maintained in the dark for 1 h, then sonicated for 30 min followed by addition of 6 mL pure hexane. Samples were placed on a RotoFlex Plus tube rotator (Sigma, France) for 10 min at room temperature and centrifuged at 7000 g for 10 min at 4 °C. After discarding the hexane layer, 2 mL of the acetonitrile-methanol-water phase were collected and stored at -20 °C until QTOF LC-MS analysis. Before analysis, 1 mL of the solution was filtered through a 0.45 µm PTFE membrane syringe 4-mm filter (Phenomenex, Torrance, USA) into an amber vial.

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For extrolite detection and quantification, 2-µL extracts were injected and compounds separated using a ZORBAX Extend-C18 column (2.1×50mm and 1.8 µm, 600 bar) maintained at 35 °C. The flow rate was set to 0.300 mL min-1 using as mobile phase: solvent A (milli-Q water + 0.1% v v-1 LC-MS grade formic acid (Carlo Erba Reagents, France) + 0.1% v v-1 ammonium formate (Thermo Fisher Scientific, Waltham, MA, USA) and solvent B (100% LC-MS grade ACN). Solvent B was maintained at 10% for the first 3 min, followed by a gradient of 10-100% of B for 45 min. Finally, solvent B was maintained at 100% for 5-min post-time. The analyses were done by ionization in both ESI+ and ESI- modes (ESI: Electrospray Ionization), retention time values (± 1 min) and the corresponding qualifier and quantifier ions were used for extrolite detection and identification as described in Supplementary Table 1. Quantification was performed using matrix matched linear ranges by preparing the standards in blank sausage casing extracts.

#### 4.2.6. *In vitro* extrolite production

*In vitro* extrolite production of one selected isolate from each Penicillium species were determined on Yeast Extract Sucrose (YES) agar medium (10 g $L^{-1}$ yeast extract, 150 g $L^{-1}$ sucrose, 0.5 g $L^{-1}$ MgSO4*7H2O, 0.006 g $L^{-1}$ ZnSO4*H2O, 0.005 g $L^{-1}$ CuSO4*5H2O, 15 g $L^{-1}$ agar) buffered at pH 4.0 with phosphate-citrate buffer. After distribution of 2 mL of YES medium into 24-wells plate, 20 µL of a 106 spores $mL^{-1}$ suspension prepared as described previously (Gillot *et al*., 2017) were deposited into each well in triplicate and incubated for 7 days at 25°C.

For extrolite extraction, 2 g of culture, comprising mycelium and medium, were weighed and homogenized in 6 mL of acetonitrile containing 0.1% formic acid (v $v^{-1}$) with an Ultraturrax T25 digital (IKA, Heidelberg, Germany) for 1 min. Then, 7.5 ml of acetonitrile containing 0.1% formic acid (v $v^{-1}$) was added to reach a final volume of 12.5 mL. After vortexing for 30 s, samples were sonicated in a sonication water bath for 15 min followed by vortexing again for 30 s before centrifugation at 5,000 g for 10 min at 4°C. Two mL of the ACN phase were collected and stored at -20°C until QTOF LC-MS analysis as described above. Before analysis, 1 mL was filtered through a 0.45 µm PTFE membrane syringe 4mm filter (Phenomenex, Torrance, USA) into an amber vial. The extracts were only analysed for the presence or absence of each extrolite and *P. nalgiovense* commercial starter culture was used as a control.

### 4.3. Results

#### 4.3.1. Culture-dependent analyses of casing mycobiota

During the course of fermentation, pH values decreased between day 0 to 15 (March batch) or day 30 (February and May batches) followed by a pH-increase up to day 50 (Supplementary Table 2). aw was stable between day 0 and 8 with a mean value of 0.95-0.96 depending on the studied batch and then decreased to 0.91 by the end of maturation (Supplementary Table 2).

Fungal counts significantly increased during fermentation in all studied batches, reaching ~6 log10 CFU $cm^{-2}$ after 4 days of ripening and increasing up to 7 log10 CFU $cm^{-2}$ after 8 days (Figure 1). From day 8 to the end of fermentation, an increase up to 7.7 log10 CFU $cm^{-2}$ was observed. Comparison of fungal growth in the three batches showed a similar trend for fungal counts in the March and May batches between days 4 and 15 while those from the February batch were significantly lower ($P<0.05$). However, after 30 days, fungal counts were similar for all batches ($P>0.05$). Noteworthy, yeast counts were generally higher than molds at the beginning of ripening (day 4 and 8), then a progressive increase in mold counts was observed (data not shown).

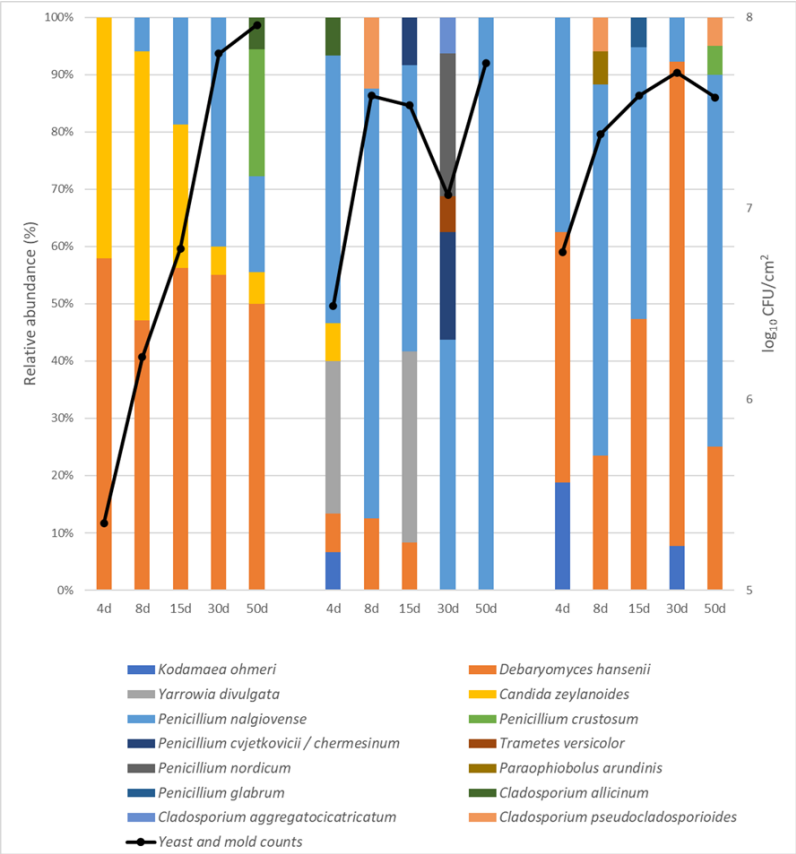


Figure 1. Fungal counts and taxa distribution in casing samples during ripening of 3 Salame Piemonte batches using a culture-dependent approach.

Concerning fungal diversity, a total of 14 species comprising 4 yeast and 8 mold species were identified (Figure 1). Yeast species included *Kodamaea ohmeri*, *Debaryomyces hansenii*, *Yarrowia divulgata* and *Candida zeylanoides*. *D. hansenii* was found in all samples and represented 60% of total yeast isolates while *C. zeylanoides* and *K. ohmeri* were isolated in February and March or March and May batches, respectively. *Y. divulgata* was only isolated in the March batch, the latter being the only one where all four yeast species were found.

Concerning filamentous fungi, five *Penicillium* species were isolated, i.e., *Penicillium crustosum*, *P. cvjetkovicii / chermesinum*, *P. glabrum*, *P. nalgiovense* and *P. nordicum*. *P. nalgiovense* was the most dominant species in all samples representing over 70% of total collected mold isolates. *P. cvjetkovicii / chermesinum* and *P. nordicum* were only isolated in the March batch. *P. glabrum* was isolated in the May batch while *P. crustosum* was isolated in February and May batches. We also identified the following *Cladosporium* species, *i.e.*, *C. allicinum* (February and March batches), *C. aggregatocicatricatum* (March batch) and *C. pseudocladosporioides* (March and May batches) which all were subdominant (<5 % of total collected mold isolates) regardless of the studied batch or sampling point. Finally, two saprobe species, i.e., *Trametes versicolor* and *Paraophiobolus arundinis*, were also sporadically isolated.

### 4.3.2. Culture-independent analyses of meat and casing mycobiota

#### 4.3.2.1. Mycobiota of casing samples during maturation

After quality filtering, a total of 932782 reads were used, with an average value of 62185 reads sample$^{-1}$, and a mean sequence length of 381 bp. Good's coverage was ~98% indicating sufficient coverage for all samples. The Good's coverage estimator was around 98% for each sample, indicating that sequencing depth was satisfactory. Significant differences in alpha-diversity index, i.e., number of observed species and Shannon index (Supplementary Figure1) were found between batches, with the May batch showing significantly higher values than the other two batches.

Thirteen taxa (9 yeast and 4 mold taxa), with a relative abundance above 0.5% in at least two samples, were observed. *Candida sake* (relative abundances ranging from 1% to 70%) and *Penicillium* sp. (ranging from 20% to 90%) were found in all samples, while the other dominant species were *D. hansenii* (relative abundances ranging from 5% to 40%), *C. zeylanoides* (relative abundance ranging around 5%) and *Y. divulgata* (relative abundances ranging from 4% to 12%) (Figure 2). OTU related to *Penicillium* sp. could not be identified to the species level.

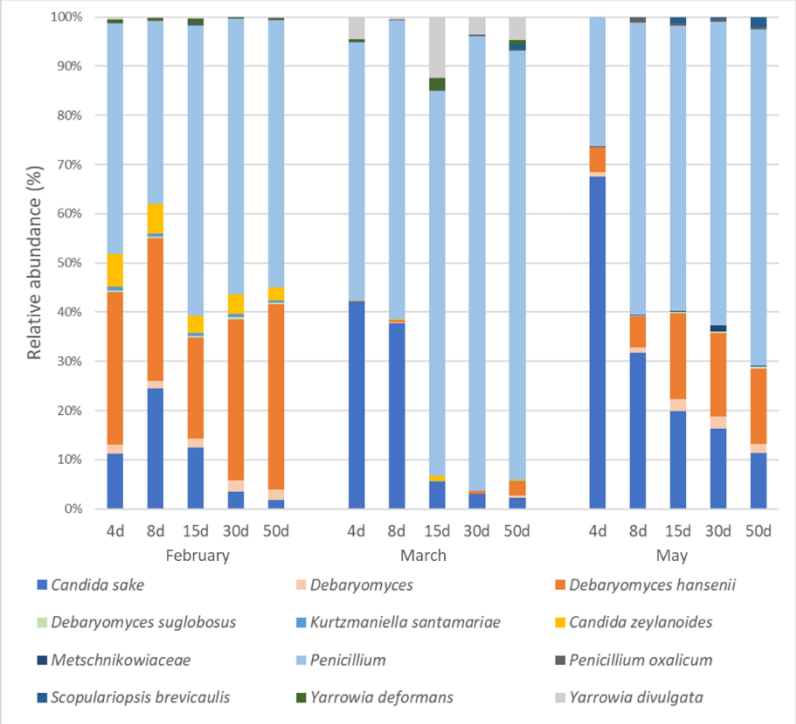


Figure 2. Taxa distribution in casing samples during ripening of three Salame Piemonte batches using a metabarcoding approach. Only taxa with relative abundances above 0.5% in at least 2 samples are shown.

PCA based on OTU table showed a sample grouping according to maturation time ($P < 0.05$) (Figure 3A) but not according to the studied batch while β-diversity analysis based on Bray-Curtis distances showed that both the batch of origin (Adonis test, $P < 0.05$, $r^2=0.1316$) and maturation time (Adonis test, $P < 0.05$, $r^2=0.2726$) shaped the casing mycobiota structure (Figure 3B). Indeed, in the PCoA, all samples collected in the February batch were grouped together while samples collected during early maturation (4 and 8 days) in March and May batches were grouped according to maturation time and those collected after 15 days were grouped according to their batch of origin. Moreover, mycobiota structure of days 15, 30 and 50 samples from the February batch were quite similar to those of the May batch. Comparison of taxa relative abundances between batches showed that *D. hansenii* and *D. suglobosus* were only associated to February and May batches while *Y. divulgata* was only observed in the March batch (Supplementary Figure 2). In addition, *C. zeylanoides* was significantly more abundant in the February batch than in the other batches. When comparing relative abundance values according to maturation time, we could observe that *C. sake* was significantly more abundant at early maturation stages (4 and 8 days) as compared to later maturation stages (Supplementary Figure 3).

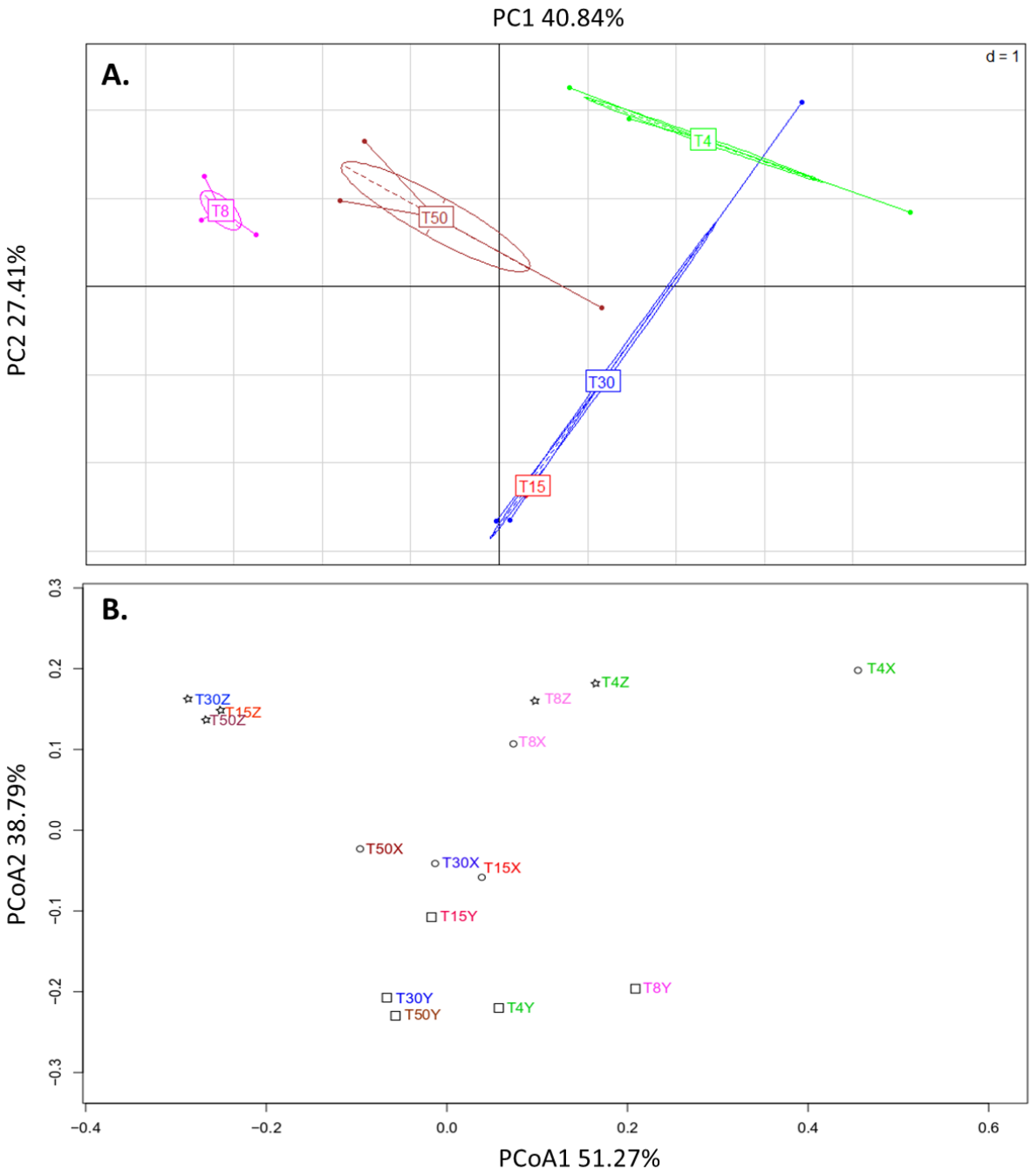


Figure 3. (A) Principal component analysis (PCA) showing casing sample mycobiota grouping according to maturation time and (B) Principal coordinates analysis (PCoA) with the Bray-Curtis index showing casing sample mycobiota grouping according to maturation time and batches.

#### 4.3.2.2. Mycobiota of meat samples during maturation

The total number of sequences obtained from meat samples throughout maturation was 805 054 raw reads. After quality filtering, a total of 72 9421 reads were used, with an average value of 48 661 reads sample$^{-1}$, and a mean sequence length of 392 bp. Good's coverage was ~98% indicating sufficient coverage for all samples. When comparing alpha-diversity index according to maturation time and batch of origin, no significant differences were observed for meat samples.

As compared to casing samples, higher fungal diversity was encountered in meat than casings with 14 and 22 yeast and mold species found in meat samples, respectively. The core mycobiota of meat samples was characterized by the presence of *Penicillium* sp. (relative abundance ranging from 10% to 80%), *Cladosporium* sp. (ranging from 1% to 7%) and *D. hansenii* (ranging from 1% to 25%) (Figure 4).

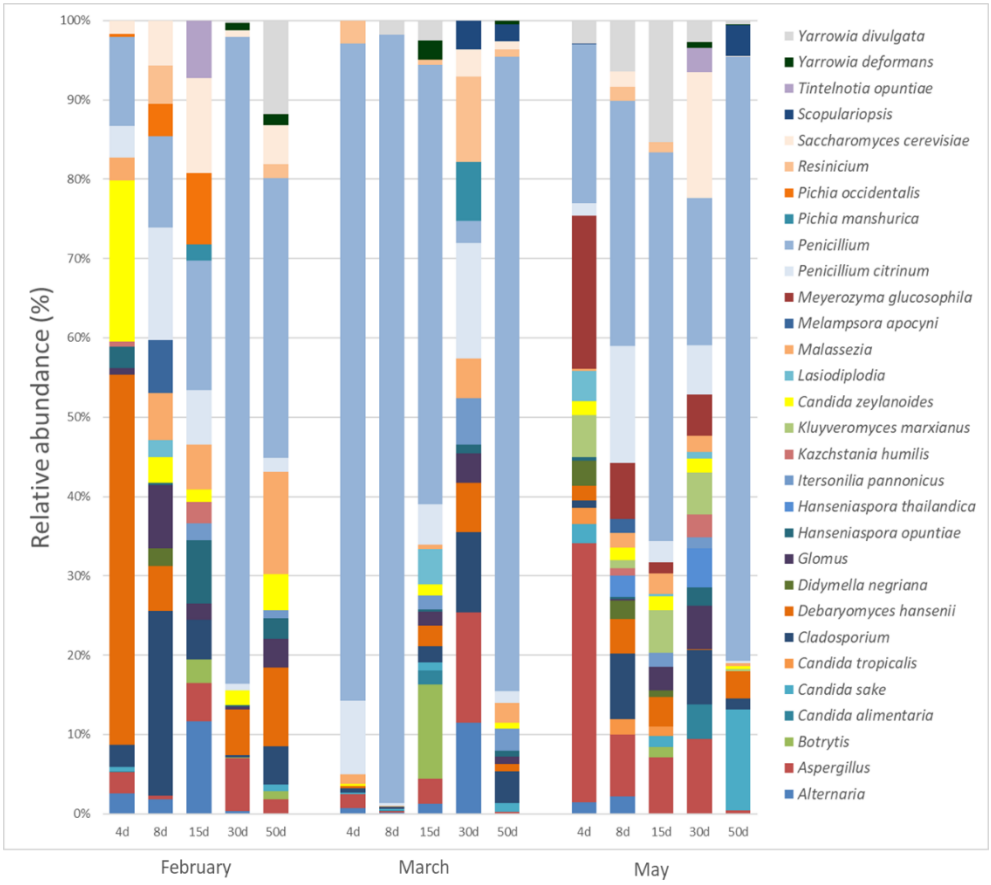


Figure 4. Taxa distribution in meat samples during maturation of 3 Salame Piemonte batches using a metabarcoding approach. Only taxa with relative abundances above 0.5% in at least 2 samples are shown.

Even though PCA did not reveal any significant sample grouping according to time or batch production date (data not shown), significant differences in species relative abundances were observed between batches or according to maturation time. Indeed, the following yeast species were only observed in the May batch, *i.e.*, *Candida tropicalis*, *Kluyveromyces marxianus* (relative abundances of ~2% on day 4, 15 and 30) and *Meyerozyma glycosophia* (relative abundances of ~3% on day 4 and of ~2% on day 8 and 30) (Supplementary Figure 4). In addition, significantly higher relative abundances of *C. zeylanoides* and *Pichia occidentalis* (relative abundance ranging from 1% to 8%) were found in samples from February (Supplementary Figure 4). In particular, a high relative abundance of *C. zeylanoides* was observed at the beginning of the fermentation (day 4) in the February batch which then decreased during the maturation period.

#### 4.3.2.3. Comparison of casing and meat mycobiota

Supplementary Figure 5 shows the species for which significant differences (FDR < 0.05) in relative abundances were found between the two sample types. Most of these species (21 out of 30 species) were only found in the meat samples and were not detected in casing samples. Except for *Aspergillus* spp., *Cladosporium cladosporioides*, and *Penicillium citrinum*, all these species had relative abundances below 1%. In contrast, *C. sake, Debaryomyces* spp. (*D. hansenii* and *D. suglobosus*) and *Penicillium* spp. were present in both sample types but were significantly more abundant in casing samples.

A correlation analysis of OTU data from casing and meat samples was performed using the spearman's rank method. As highlighted in Figure 5, presence of *C. zeylanoides*, *D. hansenii*, *S. cerevisiae* and *Pichia occidentalis* in the meat was positively correlated with the presence of *D. hansenii* and *C. zeylanoides* on casings while co-excluding pattern was shown between *Aspergillus*, *C. tropicalis*, *K. marxianus* and *M. glucosophila* in meat and *Y. deformans* and *Y. divulgata* in casing mycobiota.

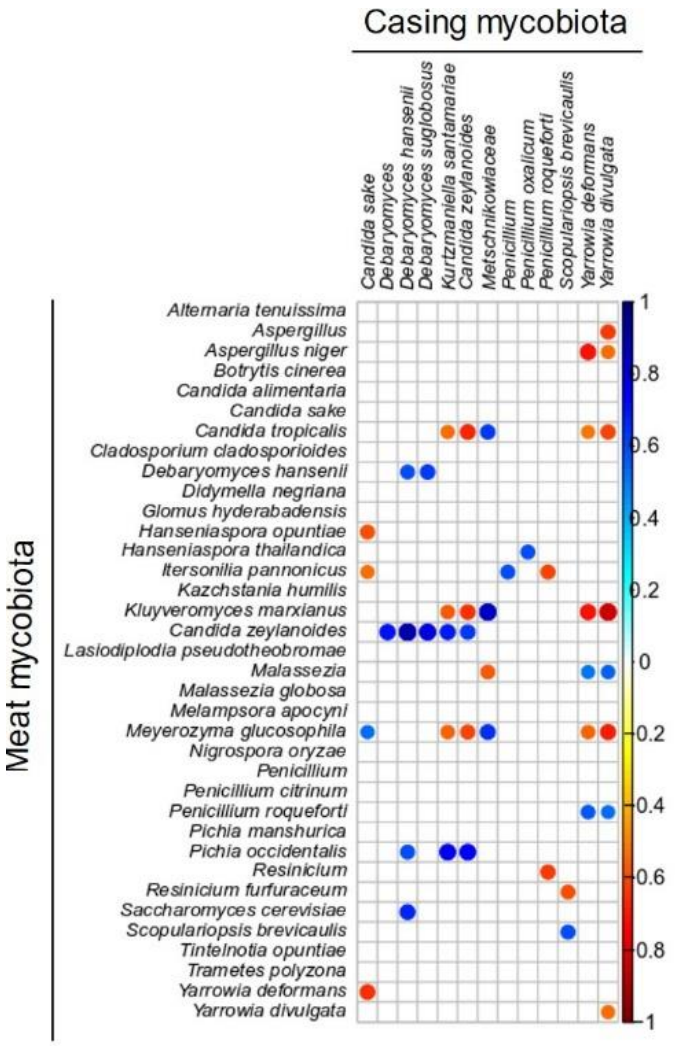


Figure 5. Spearman's correlation between the casing and meat mycobiota. (Only significant associations are shown, FDR-corrected p < 0.05). The colour intensity represents the degree of correlation where the blue color represents a positive degree of correlation and red, a negative degree of correlation.

### 4.3.3. Extrolite detection *in situ* and *in vitro*

Salami casing samples were screened for 17 different extrolites, including mycotoxins and penicillin G. All targeted mycotoxins were below their respective detection limits except for roquefortine C which was quantified at relatively low levels in March (22.86 ± 15.83 ng $g^{-1}$ at day 15 and 21.62 ± 19.91 ng $g^{-1}$ at day 30) and May (28.03 ± 25.25 ng $g^{-1}$ at day 15, 46.06 ± 26.59 ng $g^{-1}$ at day 30 and 47.99 ± 23.61 ng $g^{-1}$ at day 50) batches (Figure 6). Penicillin G was not detected in any samples. Since only trace amounts of roquefortine C were detected in the casings, the meat samples were not analysed.

Isolates belonging to *P. crustosum, P. nalgiovense, P. cvjetkovicii / chermesinum, P. nordicum* and *P. glabrum* were all screened for their ability to produce mycotoxins and Penicillin G *in vitro*. Only *P. crustosum* was confirmed to simultaneously produce roquefortine C and penitrem A although penitrem A was not detected in casing samples. All other tested strains did not produce any of the target extrolites in vitro after 7 days growth on YES agar medium including the *P. nalgiovense* commercial adjunct culture that was used for inoculating the casing surface.

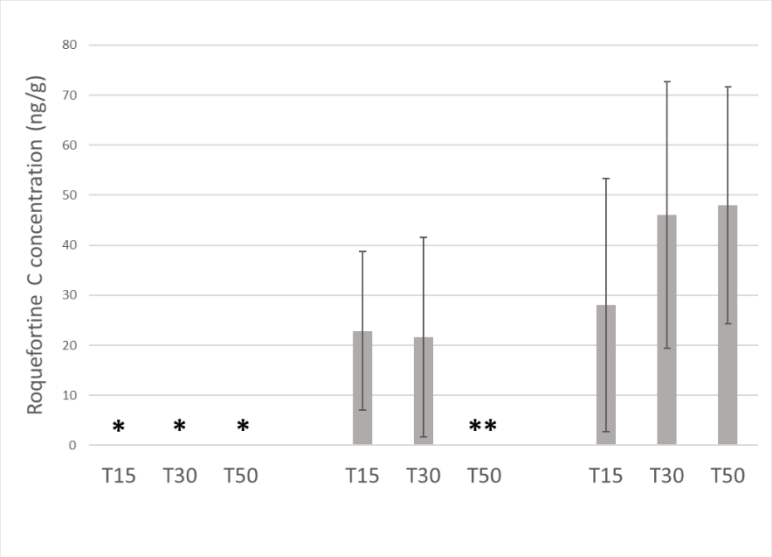


Figure 6. Roquefortine C concentration (ng/g of casing) in samples with 15, 30 and 50 days of ripening for each batch. (*mean concentration below the limit of detection (< LOD); mean concentration below the limit of quantification (< QL).

### 4.4. Discussion

In the present study, an integrative approach combining culture-dependent and metataxonomic analyses was applied to decipher *Salame Piemonte* mycobiota. Mycobiota of traditional dry-cured meat products is usually characterized by the presence of mold species belonging to the *Penicillium* genus, mainly *P. solitum, P. nalgiovense, P. chrysogenum, P. olsonii, P. commune* and *P. salamii* (Álvarez *et al*., 2020; Belleggia *et al*., 2020b; Iacumin *et al*., 2020; Zadravec *et al*., 2020; Perrone *et al*., 2015). In the present study, *P. nalgiovense* was the most dominant mold species in all batches. It is a well-known technological mold used for dry-cured

sausage production due to its positive influence on the final sensory characteristics of the product. The high dominance of *P. nalgiovense* throughout maturation is not surprising as *Salame Piemonte* casings were deliberately inoculated with a *P. nalgiovense* commercial adjunct culture. Moreover, because of its intense use in the factory and its presence on sausages during ripening, it is most likely present at high abundances in the air, e.g. drying and ripening rooms, which then act as an environmental source. Other mold species, probably transferred to the casing surface through airborne contamination or through the use of spices, included other *Penicillium* spp., *e.g. P. crustosum, P. cvjetkovicii / chermesinum, P. glabrum, P. nordicum.*and *P. oxalicum*, as well as *Cladosporium* spp. and *Scopulariopsis brevicaulis.* These species were identified at different sampling times in the different batches but were not part of the dominant mycobiota of casings reflecting proper management of air quality within the factory and the hurdle effect of the casing microbiota itself. This is especially true for *P. nalgiovense* that is often used as a protective culture against mycotoxin-producing molds in dry fermented sausages (Bernáldez *et al*., 2013). While some of these species may contribute to the organoleptic properties of *Salame Piemonte*, as reported for *C. cladosporioides* that was considered as a beneficial fungal species in Portuguese and South American sausages (Belleggia *et al*., 2020a; Parussolo *et al*., 2019a), other species are known mycotoxin producers and should be monitored.

In the present study, we were also able to unravel yeast diversity and dynamics on the casing surface and highlight the dominance of *D. hansenii* in all studied batches. This species is also well known to positively contribute to the organoleptic properties of fermented meat (Andrade *et al*., 2010; Perea-Sanz *et al*., 2020). Variations from batch to batch in yeast species abundances were observed, in particular for *D. hansenii* which was less abundant in the March batch for example, while *Y. divulgata* was only identified in the latter batch, and *C. zeylanoides* was more prevalent in the February batch. Such variations have been previously reported in other traditional fermented foods of animal origin (Belleggia *et al*., 2020a; Viljoen *et al*., 2003) and are not surprising given the fact that the yeast microbiota originates from the raw materials and the factory environment. It is worth mentioning that several species found in the present study have been rarely reported in fermented meats, such as *Y. divulgata, Trametes versicolor* and *C. sake*. The latter two species have been previously found in other dry cured meat products such as Italian San Daniele ham (Comi and Iacumin, 2013) and Italian fermented sausages (Rantsiou *et al*., 2005b). Noteworthy, *C. sake* was not isolated by our culture-dependent approach but metataxonomic analysis revealed its presence in all batches. This may be due to its slow growth and/or susceptibility to tetracycline which was used as a selective agent against bacteria for yeast and mold isolation. Indeed, a tetracycline minimum inhibitory concentration of 250-500 µg $ml^{-1}$ was reported for clinical strains of *Candida* spp. (Blanco *et al*., 2017), which is similar to the one used in the isolation medium i.e., 500 µg $ml^{-1}$. Further work would be necessary to confirm this hypothesis.

*Y. divulgata* was mainly detected in the March batch and its presence is quite unusual in this type of product in contrast to other *Yarrowia* spp., *i.e*., *Y. deformans* and *Y. lipolytica* which have been previously reported in salami products (Aquilanti *et al*., 2007; Belleggia *et al*., 2020b). The latter species is a widely reported as a spoilage agent of dairy and meat products but, at the same time, it can exert beneficial effects during the ripening of various cheeses and meat products and contributes to their organoleptic properties by aroma

compound production (Nagy *et al*., 2013; Péter G., Nagy E.S., 2019). *Y. divulgata,* which was originally described and isolated from meat products (Nagy *et al*., 2013), may have an undesirable effect on the product as this species is capable of producing high amounts of sweetener molecules, i.e., erythritol and mannitol from glycerol (Péter G. and Nagy E.S., 2019; Rakicka *et al*., 2016) with production rates higher than that of *Y. lipolytica* (Rakicka *et al*., 2016). It also possess high lipolytic activities (Nagy *et al*., 2019). It would be of interest to investigate further whether *Y. divulgata* could have a positive or negative impact on fermented meat sensorial properties.

Concerning the meat mycobiota, much higher species richness was found in meat samples throughout maturation as compared to casing samples and, in contrast to the casing mycobiota, we did not observe any significant sample grouping according to maturation time or batches. Noteworthy, fungal populations in meat were low, <3 $\log_{10}$ CFU. $g^{-1}$ as compared to those encountered in casings. The dominant species encountered on the casings from the different batches were also found in the meat, e.g., *D. hansenii*, *C. sake*, *C. zeylanoides*, *Y. divulgata* and *Penicillium* sp. although not in the same proportions. Taking into consideration the environment and the meat itself could be an important source of fungi during early maturation steps, it is also clear from these data that despite the large species richness encountered in meat, especially for yeasts, only a few species could effectively colonize the casing surface. For example, yeast species such as *Hanseniaspora*, *Kazachstania*, *Malassezia* and *Pichia* spp. and mold species such as *Aspergillus* spp. were never found on the casing despite their presence in the meat. To go further, we conducted a correlation analysis between the casing and meat microbiota using the spearman's rank correlation test. Interestingly, strong positive correlations were identified between *C. zeylanoides* in the meat mycobiota and *Debaryomyces* spp., including *D. hansenii*, in the casings. In contrast, strong negative correlations were found between *K. marxianus* in the meat and *Yarrowia* spp. in the casings. To our best knowledge, no publications related to the interactions between the above mentioned species exist and future work would be necessary to confirm these observations. Finally, considering the high fungal species richness inside the meat, it is not clear what their contribution would be, especially for yeasts, to the overall organoleptic properties of the final product. However, it cannot be excluded even though their population is limited that they participate to product typicity and the various organoleptic properties of the final product. It would therefore be of interest to investigate whether such species are metabolically active during maturation using, for example, a metatranscriptomic approach.

Concerning safety aspects, different *Penicillium* species isolated in the present study could be considered as hazardous due to their potential capacity to produce mycotoxins (Núñez *et al*., 2015). Indeed, several mycotoxin producers, including *P. crustosum* (penitrem A and roquefortine C producer as well as the antibiotic penicillin G) and *P. nordicum* (OTA producer) were isolated from the casings. Nonetheless, only trace amounts of roquefortine C were detected in samples from the March and May batches, but not in samples of the February batch. This result indicated the absence of a safety risk in relation to potential mycotoxins and penicillin G. In addition, except for *P. crustosum* which produced penitrem A and roquefortine C in vitro, none of tested isolates, including the *P. nalgiovense* adjunct culture, produced any of the targeted extrolites or penicillin G at levels above their respective detection thresholds. Given the fact that *P. crustosum* (isolated in the May batch)

was associated to the *Salame Piemonte* mycobiota, we can hypothesize that these trace contaminations were due to this species.

### 4.5. References

References of this chapter are integrated into the overall reference section (Chapter 7).

### 4.6. Research data for this article

Sequences are available at the NCBI Sequence Read Archive under the bio project accession numbers PRJNA669122.

# Chapter 5

## Autochthonous starter culture selection for Salame Piemonte PGI production

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**Abstract**

Salame Piemonte is a fermented dry cured meat typical of the Piedmont region in Italy. This product has a Protected Geographical Indication (PGI) quality label and is manufactured with a mixture of fat and fresh lean pork (*Sus scrofa domesticus* L.) using commercial starter cultures. However, the use of commercial cultures may result in a loss of the typical characteristics of artisanal foods, which are often characterized by complex microbiota. In this context, the aim of this study was to select autochthonous starter cultures that could be used during sausages fermentation.

A culture-dependent approach was applied during three different sausages spontaneous fermentation to isolate and characterize the main microbial drivers of this process. Each batch was dominated by *Pediococcus pentosaceus*, *Latilactobacillus sakei* and *Latilactobacillus curvatus* for lactic acid bacteria (LAB), while *Staphylococcus xylosus* dominated the coagulase-negative staphylococci (CNS). A total of 443 LAB and CNS isolates were further evaluated for their growth and acidification rates at different temperatures, salt concentration, nitrate reductase activity, lipolytic and proteolytic activities. After these steps, 11 strains were selected and evaluated for their ability to produce biogenic amines (BA) using a PCR assay targeting BA decarboxylase genes and for their antibiotic resistance pattern. Selected strains (two *P. pentosaceus*, two *L. sakei* and one *S. xylosus* strains) were then used for pilot-scale production of Salame Piemonte with 7 different strain combinations. Sausages were subjected to microbial safety and hygiene evaluation, metataxonomic, volatilome and consumer test analyses. Overall, the use of *P. pentosaceus* and *S. xylosus* autochthonous starter cultures significantly improved product sensorial properties as compared to the product obtained with commercial starter cultures.

### 5.1. Introduction

The microbiota of fermented meat products is composed of useful and specific microbial communities which individual members and their interactions positively contribute to the fermentation process by providing their safety and distinct organoleptic properties (Franciosa *et al*., 2018). This microbiota is first established during animal slaughtering and is then enriched by a specific house-microbiota from each facility environment (Ammor *et al*., 2005; Stellato *et al*., 2016). This typical house microbiota is a source of microorganisms that may participate in the fermentation process and contribute to product typicality.

Dry-cured fermented sausages are usually produced with minced meat (mix of fat and slim parts) plus nitrates, nitrites and seasoning that are homogeneously mixed and then stuffed into a natural or artificial casing (Cruxen *et al*., 2019; Franciosa *et al*., 2018). The sausages are then placed in a maturation room where the fermentation process starts. All the different steps and ingredients characterize the fermentation process, which is driven by specific wild microbes (Baka *et al.*, 2011) that, by complex chemical and physical reactions, give the sensorial properties (firmness, flavour and colour) to the final product (Ammor and Mayo, 2007; Stellato *et al*., 2016).

Nowadays, at industrial level, the use of starter cultures has replaced the practice of spontaneous fermentation produced by unspecified microbiota, to provide standardized characteristics for the final product and to avoid food losses by microbial spoilage (Cruxen *et al*., 2019). According to the definitions present in literature, "a starter culture is a preparation which contains living microorganisms, previously characterized as safe and exhibiting desired metabolic activity, used to modify the sensory properties of the food" (Ammor *et al*., 2005; Bourdichon *et al*., 2012). Most of the available commercial meat starter cultures contain a mixtures of lactic acid bacteria (LAB) and coagulase negative staphylococci (CNS) (Ammor and Mayo, 2007; Hu *et al*., 2019). The main function of LAB in meat fermentations is to obtain a rapid pH drop, which increases the product safety, stability and shelf life of the final products (Cardinali *et al*., 2018). CNS allow the formation of the typical sensory characteristics and colour of the sausage, due to their lipase, protease and nitrate reductase activities (Cardinali *et al*., 2018; Chen *et al*., 2017). This type of fermented sausage is also a perfect substrate for fungal growth and many fungal species are able to colonize dry fermented sausages. *Debaryomyces hansenii* and *Penicillium nalgiovense*, are major species and are often used as starter cultures for casing inoculation (Murgia *et al*., 2019; Sunesen and Stahnke, 2003). This mycobiota also plays an important role in sausage sensory characteristics. (Sonjak *et al.*, 2011).

Strains composing commercial starter cultures are usually isolated from the wild microbiota of spontaneous fermentations, since they are well adapted to the ecological niche in which they are intended to be used (Baka *et al*., 2011; Cruxen *et al*., 2019). An accurate strain-level characterization and selection is also necessary because strains belonging to the same genus can have a different impact on the final product characteristics. Besides, the strain selection process should be designed based on the role of the microbes during the fermentation process. Furthermore, regarding safety aspects, strains used in food industry as starter cultures should not be pathogenic, possess biogenic amine potential production and acquired antimicrobial resistance (Álvarez-Cisneros and Ponce-Alquicira, 2018; Laslo *et al*., 2020).

It has been shown that the use of autochthonous strains significantly improves product sensorial properties as compared to commercial starter cultures and that native strains contribute to create the distinct final properties of a typical regional fermented product (Baka *et al*., 2011; Cruxen *et al*., 2019). In this context, the aim of this study was to select autochthonous starter cultures that could be used as a new starter culture for Salame Piemonte manufacturing.

To reach our objectives, we analyzed three different batches of spontaneously fermented sausages produced at different months in the same factory following the same recipe. From each fermentation batch, we isolated and identified LAB and CNS, screened them for their technological properties and evaluated their safety in order to select the most suitable strains or strain combinations to compose different starter formulations. These starter formulations were then used to produce different pilot-scale batches of Salame Piemonte which were compared in terms of metataxonomic composition, volatilome and sensorial properties through consumer test analyses.

## 5.2. Material and methods

### 5.2.1. Sample collection from spontaneous fermented sausages

Spontaneous fermented sausage samples were collected at time 0 (meat plus seasoning) and from sausages after 4, 8, 15, 30 and 50 days of fermentation from three different batches of Salame Piemonte PGI produced in February, March and May 2018 according to the detailed experimental procedure reported elsewhere (Franciosa *et al*., unpublished). At each sampling point, microbial counts, water activity ($a_w$) and pH determination were performed. Briefly, aliquots of about 10 g from each sample were collected from the core and individually homogenized with 90 ml of Ringer's solution (Oxoid, Milan, Italy) for 2 min in a stomacher (LAB blender 400; PBI, Italy). Decimal dilutions in quarter-strength Ringer's solution were prepared and spread on the following media: de Man-Rogosa-Sharpe agar (MRS; Oxoid) for lactic acid bacteria (LAB) incubated at 30°C for 48 h in anaerobic condition; mannitol salt agar (MSA; Oxoid) for coagulase-negative staphylococci (CNS) incubated at 37°C for 24 h; violet red bile agar (VRBA; Oxoid) for *Enterobacteriaceae* incubated at 37°C for 24 h; Harlequin Listeria Chromogenic Agar (Lab M, Milan, Italy) upon sample enrichment in Fraser broth (Oxoid) at 37°C for 24 and 72 h for *Listeria monocytogenes*; Kanamycin Aesculin Azide Agar (KAA, Oxoid) for *Enterococci* at 37°C for 48 h; Xylose-Lysine-Desoxycolate Agar (XLD, LAB M) and Brilliant Green Agar (BGA, LAB M) after sample pre-enrichment in Buffered Peptone Water (BPW, Lab M) and Soya Peptone Rappaport Vassiliadis (RVS, Lab M) for *Salmonella* spp. incubated at 37 ° for 24 h; Reinforced Clostridial Agar (RCA, Biolife) for *Clostridia* spp; Tryptone Bile X-GLUC (TBX, Oxoid) at 37°C for 48 h to detect *Escherichia coli* colonies and Baird Parker Agar Base (BP, Oxoid) plus egg yolk tellurite emulsion at 37°C for 48 h for *Staphylococcus aureus*.

The pH was measured by a pH probe of a digital pH meter (micropH2001; Crison, Barcelona, Spain) while $a_w$ was measured with a calibrated electric hygrometer (HygroLab; Rotronic, Bassersdorf, Switzerland) according to the manufacturer's instructions.

About fifteen colonies from MRS and MSA media at each sampling point from each batch were randomly isolated and purified. As previously described by Franciosa *et al*. (unpublished), isolates were subjected to

DNA extraction, PCR (rep-PCR) with the $(GTG)_5$ primer and cluster analysis as already reported (Ferrocino *et al*., 2017). After cluster analysis, 2 isolates from each cluster at 80% of similarity were selected and subjected to identification. LAB and CNS identification was performed by amplifying the 16S rRNA gene (Ferrocino *et al.*, 2017; Weisburg *et al*., 1991). All the bacteria isolates were stored frozen (-20 °C) in MRS broth or BHI broth supplemented with 30% glycerol.

### 5.2.2. Strain physiological characterization

Growth curves were reconstructed for each isolate in the following conditions. LAB isolates were grown in MRS broth using four different temperatures (30, 23, 15 and 10°C) and different NaCl concentrations (2, 3 and 4% v/v), while CNS isolates were grown in BHI broth in the same conditions. The acidification rate at different temperatures (30, 23, 15 and 10°C) was also measured.

Briefly, an overnight culture of each isolate was inoculated at 2% (v/v) in 200 µl of the different media described above. Growth curves were obtained by using the 96 well Bio-Tek Elx808 microtiter plate reader at 630 nm (Bio-Tek Instruments, Inc. VT, USA) with reads performed every 30 min till the plateau. Spectrophotometric data were processed in the R environment with the package *Growthcurver*. The outputs were: K values, corresponding to the maximum possible population size in a particular environment; $n_0$ values, population size at the beginning of the growth curve; r values, intrinsic growth rate of the population ($\mu_{max}$). The latter value was used to express the growth rate of each isolate. A filtered table (K values > 0.900) was then used in order to eliminate the outliers (strains that were not at plateau at the end of measurements). Non-inoculated control samples were included in the readings and blanked data were used for the modelling. Each isolate was analysed in triplicate.

CNS isolates were tested for their lipolytic activity on Spirit Blue Agar plates supplemented with a mixture of olive oil and Tween 80 (Ercolini *et al.*, 2010), proteolytic activity on skim milk agar (1% skim milk, 1.5% agar); nitrate reductase activity by spectrophotometric assay (Casaburi *et al*., 2005). Lipolytic and proteolytic activity were evaluated by the detection of clear zones around the colonies after 24h at 37°C. *L. sakei* and *S. xylosus* strains composing the commercial starter culture currently used by the product manufacturer were analysed and used as a control.

### 5.2.3. Safety evaluation

Based on the physiological and molecular results, selected strains (8 LAB and 2 CNS) were evaluated for their safety by detection of biogenic amine genes and screening for antibiotic resistance. After DNA extraction from 1 mL overnight bacterial cultures with the FastDNA Spin Kit (MP Biomedicals, France) according to the manufacturer's instructions, detection of four biogenic amine genes: histidine decarboxylase (*hdc*), tyrosine decarboxylase (*tyrdc*), putrescine decarboxylase (*odc*) and agmatine deiminase (*agdi*) genes was performed by multiplex PCR as described by Coton *et al.* (2010, 2018). An uniplex PCR for each BA gene was performed to confirm the results of the multiplex. All strains that showed the presence of biogenic amine genes were excluded for future food applications and the remaining strains (4 LAB and 2 CNS) were tested for their antibiotic resistance. Antibiotic resistance was determined for 12 different antibiotics (kanamycin, streptomycin, tetracycline, erythromycin, clindamycin, chloramphenicol, ampicillin, neomycin, vancomycin, trimethoprim, oxytetracycline, rifampicin) according to the ISO 10932 IDF 223 International Standard (2010-06-15) (Coton *et al*., 2018). *Lactiplantibacillus plantarum* (LMG6907) was used as reference strain as specified by the ISO standard. Each strain was assayed in triplicate in the microplates arranged as explained in the ISO guideline and the MIC was read after 48h as the first well where no growth was visible. The growth was verified by an automatic Multiskan FC plate reader (Thermo Scientific, Paris, France) set to 620 nm. The final results were expressed as minimum inhibitory concentrations in micrograms per milliliter and the values were compared to the EFSA breakpoints (Food and Authority, 2012) and literature data (Danielsen and Wind, 2003). Only strains that did not show any antibiotic resistance beyond known natural ones (4 LAB and 1 CNS strains) were considered as safe and used for meat inoculation at a pilot-scale.

### 5.2.4. Starter formulation and meat inoculation

Fermented sausages were produced at a meat processor in the province of Turin (Piemonte Region, Italy) and prepared using five different autochthonous strains organized in seven different autochthonous starter cultures, (ASC) (Table 1). The sausage formulation (20 Kg of meat mixture for each ASC) included pork meat (lean from the shoulder and fat from the belly), salt (maximum 3%), pepper (maximum 0.4%), aromatic plants and spices, *i.e*., garlic and cloves, whole, crushed or infused with wine and nutmeg. After chopping and mixing the ingredients, the mixture was divided into batches according to each autochthonous starter formulation. Each batch was individually inoculated with about $10^7$ CFU/g starter. At the same time all the sausages were also inoculated on the casing surface with a commercial starter culture of *P. nalgiovense* how usually perform for the classic Salame Piemonte production.

The cutter and filler were cleaned and disinfected before the pilot-scale production and between each different batch to avoid contamination. Sausages stuffed into a natural casing with a diameter of 6 cm were placed in the ripening room with the same condition used for the industrial production (Franciosa *et al*., unpublished), for fifteen days. A total of 12 sausages of about 34 cm length and 655 g weight were obtained for each ASC as described above. At the same time, a control sausage was prepared by using a commercial starter culture composed by a mixture of *L. sakei* and *S. xylosus* ($10^7$ CFU/g) and used in all the analyses as control.

Table 1. Strain composition of the seven autochthonous starter culture (ASC).

| | *P. pentosaceus* | *L. sakei* | *S. xylosus* |
|---|---|---|---|
| **ASC 1** | S4XNM | - | S8HS |
| **ASC 2** | S8QM | - | S8HS |
| **ASC 3** | S8QM+S4XNM | - | S8HS |
| **ASC 4** | - | S29ZEM | S8HS |
| **ASC 5** | - | S29BM | S8HS |
| **ASC 6** | - | S29BM+S29ZEM | S8HS |
| **ASC 7** | S4XNM | S29ZEM | S8HS |

### 5.2.5. Physico-chemical and microbial analyses of the inoculated sausages

Samples were collected after 0, 2, 12 and 15 days of fermentation for physico-chemical and microbiological analyses. At each sampling time, three sausages were analysed for each ASC.

For each sausage 10 g of meat were homogenized in 90 ml of Ringer's solution (Oxoid, Milan, Italy) for 2 min in a stomacher (LAB blender 400; PBI, Italy), aliquots of this solutions were used for pH and microbial count determinations. Lactic acid bacteria (LAB) were enumerated on de Man-Rogosa-Sharpe (MRS; Oxoid) agar supplemented with 250 mg/L cycloheximide after incubation at 30°C for 48 h in anaerobic conditions and coagulase-negative staphylococci (CNS) on mannitol salt agar (MSA; Oxoid) incubated at 37°C for 24 h. Detection of *Listeria monocytogenes* was also performed by means of the Harlequin Listeria Chromogenic Agar (Lab M) upon sample enrichment in Fraser broth (Oxoid) at 37°C for 24 and 72 h.

### 5.2.6. Molecular and metataxonomic approach

Around 9 colonies from both MRS and MSA media at each sampling point were isolated and purified. They were used to track the presence of the inoculated strains during fermentation using rep-PCR as described previously by comparison of their rep-PCR patterns with those of inoculated strains.

For the metataxonomic approach, total DNA was extracted from samples at the end of fermentation and used for metataxonomic analyses by amplifying the V3-V4 regions of the 16S rRNA gene following the conditions described by Klintword *et al*. (2013). PCR products were purified, tagged, pooled and sequenced following Illumina guidelines. Sequencing was performed on a MiSeq instrument (Illumina) with V3 chemistry, and 250-bp paired-end reads were generated. Raw files (.*fastq*) were imported in QIIME 2 software (Bolyen *et al*., 2019). Primers were removed by using Cutapt, obtained reads were denoised by DADA2 algorithms (Callahan et al., 2016) by using the q2-dada2 plugin in QIIME 2 in order to remove chimeric sequences and low quality reads in order to obtain the ASVs.

The samples were classified taxonomically using the QIIME2 feature-classifier against the *Greengenes* 16S rRNA gene database v. 13.8. ASV with less than five read counts in at least two samples were excluded from

further analysis to increase the confidence of sequence reads and reduce bias resulting from possible sequencing errors. Normalization of sequence reads was done at the lowest sequence/sample by verification with an equal representation of 10,000 sequences per sample. Qiime2 diversity plugin was used to perform alpha and beta diversity analyses.

#### 5.2.7. Volatilome analyses of fermented sausages

The volatilome composition of the final product was performed by headspace (HS) solid-phase microextraction (SPME) and analysed by gas chromatography-mass spectrometry (GC/MS). Briefly, 3 g of sample were placed in 20 ml vials with the internal standard: 10 µl of 2-octanol in ultrapure water (333 ppb as final concentration). The analyses were carried out using a GC-2010 gas chromatograph equipped with a QP-2010 Plus quadruple mass spectrometer (Shimadzu Corporation, Kyoto, Japan) and a DB-WAXETR capillary column with 30 m length, 0.25 mm internal diameter, 0.25 µm film thickness (J&W Scientific Inc., Folsom, CA). We measured the *m/*z peak area of the quantifier ion in relation to the *m/z* quantifier ion of the added internal standard to obtain a semiquantitative result (µg/kg) for each identified compound (Ferrocino *et al*., 2018).

#### 5.2.8. Sensory evaluation of the inoculated sausages

To assess the sensory acceptability of sausage samples at the end of ripening, a consumer test was performed. A total of 20 consumers of sausages (7 male and 13 female participants; aged 28 to 56 years) voluntarily participated in the sensory evaluation. The panelists were served with a slice of each sausage randomly numbered with a glass of water to clean their mouth between each sample. They evaluated external appearance, texture, colour, flavour and global appreciation. For each sample, panelists completed a table modified from the one described by Chiavari *et al*. (2007). The results were elaborated and plotted using a radar graph generated in the R environment.

#### 5.2.9. Statistical analyses

A one way-analysis of variance (ANOVA) was used to detect significant differences among means followed by a Duncan's multiple range test for mean comparison of microbial counts, physico-chemical parameters, alpha diversity parameters and ASV abundance using the SPSS 17.0 program for Windows (SPSS Inc., Chicago, IL, USA) or QIIME2 as appropriate.

### 5.3. Results

#### 5.3.1. Bacterial counts and microbiota composition of spontaneously fermented sausages

pH values decreased as a function of fermentation time showing some differences between the three analyzed batches (Supplementary Table 1, $P<0.05$). In particular, in February, March and May samples, the initial pH values were 6.49, 6.03 and 5.99 reaching 5.29, 5.72 and 5.35 at the end of the fermentation process, respectively. Noteworthy, at the end of the fermentation process, March samples showed the highest pH value and February samples the lowest, despite their highest initial pH values (Supplementary Table 1, $P<0.05$). For $a_w$ values, no significant differences in the three batches were observed.

In all analyzed batches, LAB counts rapidly increased from ~3 $\log_{10}$ CFU/g to 9 $\log_{10}$ CFU/g in the first eight days of ripening and then remained constant till the end (Supplementary Table 1). May samples showed the highest LAB population at the end of fermentation (9.71 $\log_{10}$ CFU/g) as compared with the 2 other batches (Supplementary Table 1 $P<0.05$). CNS counts remained relatively constant during ripening with around 3 $\log_{10}$ CFU/g, the highest value being observed at the end of February fermentation (4.23 $\log_{10}$ CFU/g) (Supplementary Table 1, $P<0.05$). For *Enterobacteriaceae*, we observed that after two weeks of ripening the count decreased below the detection limit in the March and May batches, while it reached about 1.59 $\log_{10}$ CFU/g at the end of ripening in samples from February (Supplementary Table 1). *Escherichia coli*, *Enterococcus* spp., *S. aureus* and *Clostridium* spp. counts were below detection levels (< 2 $\log_{10}$ CFU/g) while *L. monocytogenes* and *Salmonella* spp. were not detected in any sample.

A total of 443 isolates comprising 224 LAB and 219 CNS isolates were obtained from the three different batches and further identified. As shown in Figure 1, LAB isolates were dominated by *L. sakei* (177 out of 224 isolates with 58, 46 and 73 isolates from February, March and May batches, respectively), *P. pentosaceus* (19 out of 224 isolates with 17 and 2 isolates from February and May batches, respectively) and *Latilactobacillus curvatus* (26 out of 224 isolates only originating from the March batch). In addition, 1 isolate each of *Lactiplantibacillus plantarum* and *Loigolactobacillus coryniformis* were isolated from March samples. For CNS, 5 species were identified, i.e., *S. xylosus* (201 out of 219 isolates with 71, 67 and 63 isolates from February, March and May batches respectively), *Staphylococcus succinus* strains (6 isolates including 1 and 5 from February and May batches, respectively), *Staphylococcus equorum* strains (6 isolates including 3 and 3 from February and March batches), *Staphylococcus carnosus* (5 isolates from May samples) and *Staphylococcus saprophyticus* (1 isolate from March batch) (Figure 1).

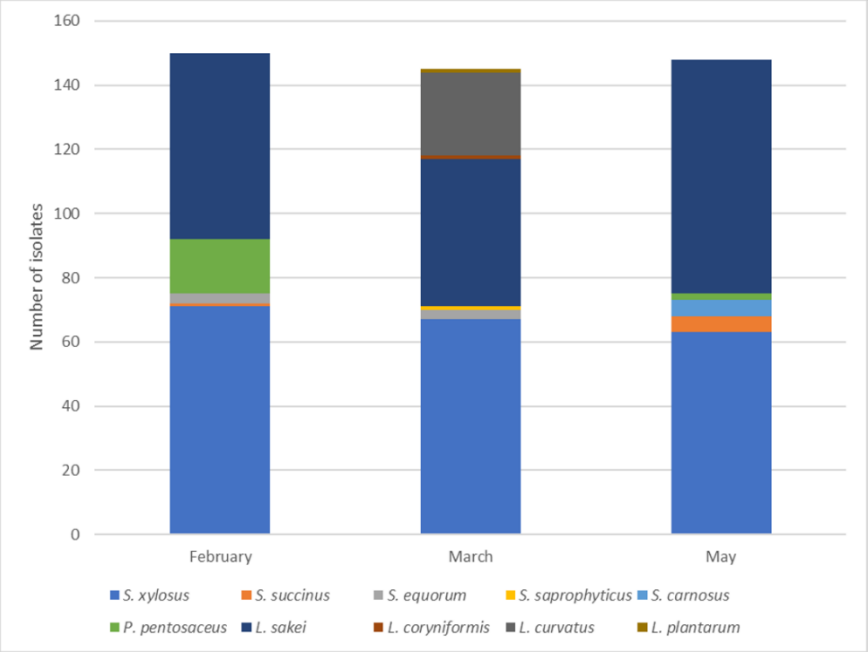


Figure 1. Diversity of bacterial isolates from 3 batches of spontaneous fermented sausages.

### 5.3.2. Physiological characterization of isolates

LAB and CNS isolates were screened for their physiological properties. Even though the results showed that isolates harboured different physiological characteristics (data not shown), we were able to discriminate the three fermentation batches based only on the acidification rate at all tested temperatures of the isolates from which they originated (Supplementary Figure 1). An ANOSIM test confirmed this observation ($P<0.001$) as isolates from February batch were clearly separated from the other 2 batches while isolates of the latter batches did not differ significantly in their acidification rate as their respective confidence ellipse overlapped. In more details, LAB isolates from the February batch displayed higher acidification rate at 20°C than those from the other two batches. In contrast, lower acidification rate at 15°C and lower growth rate in the presence of NaCl (Supplementary Table 2A, $P<0.05$) was observed for isolates belonging to the February batch (Supplementary Table 2A). By comparing the performance of the three species groups of LAB isolates (Supplementary Table 2B), we observed that: *L. curvatus* isolates had low growth rates at 20 and 30°C and displayed a generally low acidification rate. *P. pentosaceus* showed the highest acidification rate at all tested temperatures (Supplementary Table 2B) while *L. sakei* showed, in general, intermediate characteristics as compared with the other two species.

Almost all tested LAB (207 out of 224 isolates) were able to grow in the presence of 2% NaCl and their growth rate decreased as NaCl concentration increased. One-hundred and forty-three and 55 were able to grow in the presence of 3% and 4% NaCl, respectively. CNS strains showed less tolerance to salt, only 27 strains of 219 isolates were able to growth in the presence of 4% NaCl.

The growth ability in relation to temperature showed that a total of 37 LAB isolates did not grow at 30°C, 44 at 20°C, 145 at 15°C and 198 at 10°C; while a total of 123 CNS isolates didn't grow at 30°C, 99 at 20°C, 90 at 15°C and 59 at 10°C.

### 5.3.3. Isolates selection

Autochthonous starter LAB were selected based on their growth rate at 20°C and 15°C. In details, we considered strains with good performance those that showed μmax at 20°C and 15°C ranging from 0.30 and 0.12 and 0.16-0.05, respectively. These temperature ranges were chosen according to the PGI product specification (Official Gazette of the Italian Republic, nr 184, 10 August 2015), since temperature during the first week of the fermentation process gradually decreases from ~25°C to ~15°C which is associated with a rapid increase in microbial count during the first week of fermentation. Regarding acidification properties, selected strains were chosen based on final pH values reached at 15-10°C with values comprised between 6.01 and 5.52, since the bacteria have to show high acidification rate in the first week of fermentation and a low acidification activity in the later stages (at 15°C). Among CNS isolates, we selected isolates with lipolytic, proteolytic and nitrate reductase activities and a rapid growth at 20 and10°C.

After this selection, rep-PCR fingerprints of selected isolates were compared in order to select only those with different rep-PCR patterns (data not shown). At the end of the selection process, selected bacteria were five *P. pentosaceus* strains (C1M, S4NM, S8QM, S4XNM, S45XEM), three *L. sakei* strains (S29BM, S15ZGM, S29ZEM) and two *S. xylosus* strains (S8HS, S29XIS).

### 5.3.4. Safety evaluation of selected isolates

Safety evaluation of the latter 10 strains was performed through detection of biogenic amine (BA) genes and screening for antibiotic resistance. None of the CNS strains showed any specific bands for the targeted biogenic amine genes by multiplex PCR. In contrast, targeted genes were detected in several LAB strains by both multiplex and simplex PCR, *i.e.*, *P. pentosaceus* C1M and S4NM were positive for *tyrdc* gene, *P. pentosaceus* S45XEM was positive for *odc* gene while *L. sakei* S15ZGM was positive for *agdi* gene.

The minimum inhibitory concentration (MIC) for 12 antibiotics was determined for 6 strains which were identified as non-BA-producers (Supplementary Table 3). Overall, intermediate MIC values were found for all antibiotics and strains. As expected, all LAB strains were resistant to vancomycin. Both *L. sakei* strains (S29BM and S29ZEM) did not show any antibiotic resistance above EFSA cut-off values while *P. pentosaceus* S8QM showed MIC values slightly above EFSA cut-off values for kanamycin and streptomycin while strain S4XNM did not. Both tested *S. xylosus* strains showed resistance to chloramphenicol with S8HS displaying a resistance close to the EFSA cut-off value.

Based on the above-mentioned results, *P. pentosaceus* S8QM and S4XNM, *L. sakei* S29BM and S29ZEM and *S. xylosus* strain S8HS were selected for pilot-scale fermented sausage production.

### 5.3.5. Sausage production, microbial counts and pH of inoculated sausages

Seven autochthonous starter cultures (ASC) were then tested for sausage manufacture at a pilot-scale (Table 1). Microbiological analyses showed an absence of *L. monocytogenes* detection in all samples. The final pH value of the control sausages (made with commercial starter cultures) was 5.21 and those of sausages produced with the seven ASC were in the same range without any significant differences, although the seven batches did not follow a similar acidification trend (Supplementary Table 4, $P<0.05$). Indeed, initial pH values were different between batches with ASC 3 showing the lowest pH value (5.76) while for other batches initial pH values were ~6 (Supplementary Table 4). After two days of fermentation, a pH decrease to ~5 was observed for ASC 1, 2 and 3 and control samples while for the other tested ASC, an unexpected pH increase occurred. After 12 days of fermentation, the opposite situation was observed with a slight pH increase in ASC 1, 2, 3 and control samples while for the other samples showed pH values dropped to 5.25-5.42. At this sampling time, the samples could be divided in two groups, i.e., ASC 1, 2, 3, 4, 7 and control samples with pH values around 5.2 ASC 5 and 6 samples with pH value around 5.4. During the last three days of fermentation, ASC 5 and 6 pH values reached 5.2 while pH values of other samples remained constant (Supplementary Table 4).

ASC 1, 2 and 3 samples, showed CNS counts never lower than 8 $\log_{10}$ CFU/g. The other four ASC showed lower values, starting from 7.5 $\log_{10}$ CFU/g with a slight decrease until the end of the fermentation to 6.7 $\log_{10}$ CFU/g.

A similar trend was observed for LAB counts at the beginning of fermentation. Indeed, after two days of fermentation, LAB counts in ASC 4, 5, 6 and 7 samples were significantly lower than in the other samples, including the control (Supplementary Table 4, $P<0.05$). After 12 days of fermentation, no significant differences were observed between the inoculated sausages except for the ASC 7 (*P. pentosaceus* S4XNM and *L. sakei* S29ZEM) for which LAB count was below 9 $\log_{10}$ CFU/g (Supplementary Table 4, $P<0.05$). At the end of the fermentation the LAB count of ASC 7 remained the lowest (Supplementary Table 4, $P<0.05$).

For all tested conditions, the presence of selected autochthonous starters at the end of fermentation was confirmed by rep-PCR (data not shown).

### 5.3.6. Metataxonomic composition

A metataxonomic approach was used in parallel to analyze microbial diversity in the final product of each trial, in order to investigate the impact of inoculated strains on the fermented meat microbiota.

Significant differences in alpha-diversity indexes, i.e., chao-1, number of observed species and Shannon index were found between the different samples (data not shown). In particular, samples produced with ASC 4 showed significantly lower values for the Shannon index than the other ASC inoculated samples ($P<0.05$). Alpha-diversity indexes of control samples the lowest values as compared to all other tested conditions.

Comparison of ASV relative abundances between sausages at the end of the fermentation process and made with the different ASC confirmed dominance of the inoculated species (Figure 2). Other minor species, i.e., *Lactococcus lactis*, *Weisella viridescens*, were present at significantly higher relative abundances in samples inoculated with ASC 5, while *Loigolactobacillus coryniformis* was significantly more prevalent in the control as compared to other tested conditions. *Lactococcus garvieaeas* was only observed in sausages produced with ASC 4, 5 and 6 (Figure 2). *Kocuria rhizophila*, and *Macrococcus caseolyticus* were only identified, although with low relative frequency, in samples inoculated with *P. pentosaceus* (ASC 1, 2 and 3). *Weisella bombi* was more abundant in sausages inoculated with *P. pentosaceus* (ASC 1, 2 and 3) (Figure 2).

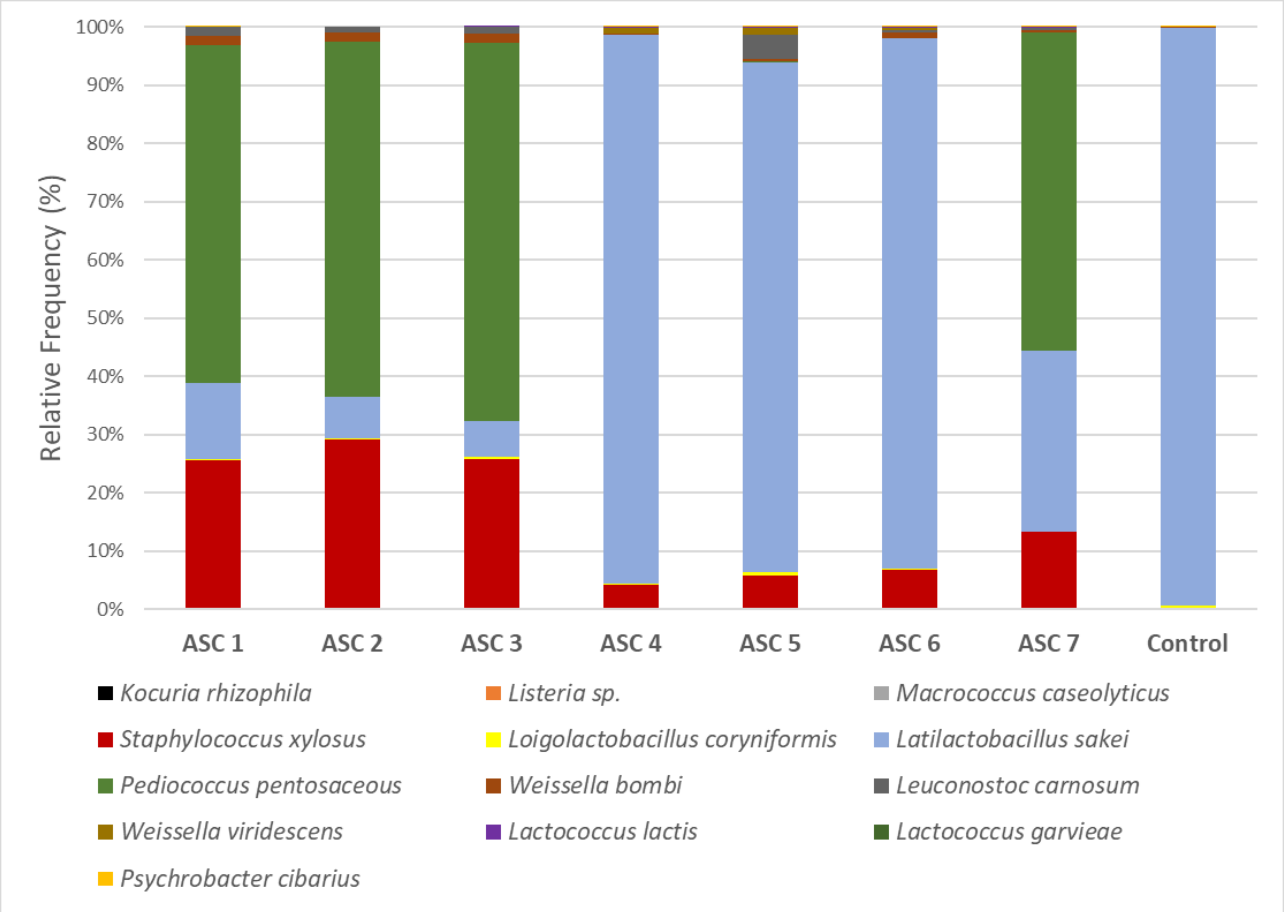


Figure 2. Amplicon sequence variant relative abundance (%) in Salame Piemonte sausages inoculated with different autochthonous starter culture (ASC) using a metabarcoding approach.

Figure 3 shows the relative abundances of species composing the different tested ASC as well as that of *Listeria* spp. Overall, for each tested condition, ASV corresponding to species initially present in the starter culture were detected at relatively high abundances with the exception of the control sausage in which *S. xylosus* was not detected at all. Relative abundances of *L. sakei* were the highest (ranging from 87% to 94%) in sausages inoculated with ASC 4, 5, 6 and control which were all deliberately inoculated with *L. sakei* while it was at an intermediate level for sausages produced with ASC 7, composed of both *P. pentosaceus* and *L. sakei*. Noteworthy, despite *L. sakei* was not deliberately inoculated in ASC 1, 2 and 3 sausages, its relative abundance ranged between 6% and 13%. On the other hand, *P. pentosaceus* relative abundances was high (ranging from 55% to 65%) in sausages produced with ASC 1, 2, 3 and 7, all of which containing that species, whereas it was not detected in other samples. Concerning *S. xylosus* which was inoculated in all tested conditions, it was detected, except for the control sausage, at abundances ranging from 4% and 29% with a higher abundance in sausage samples inoculated with *P. pentosaceus*. Interestingly, for all sausages which were not inoculated with *L. sakei*, ASV related to *Listeria* spp. were detected with relative abundances around 0.1%.

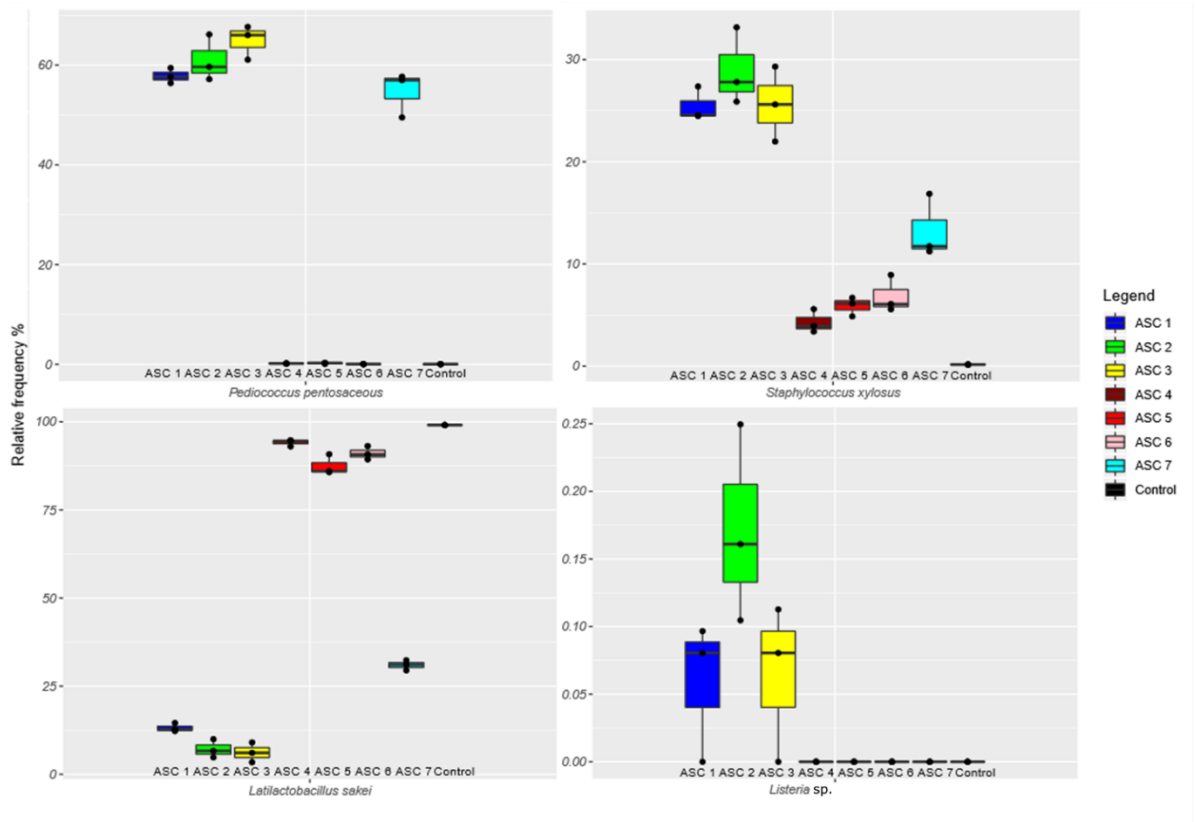


Figure 3. Boxplots showing the relative frequency of *P. pentosaceus*, *S. xylosus*, *L. sakei*, and *L. monocytogenes* present in the sausages microbiota of the seven different autochthonous starter culture (ASC) studied sausages. ASCs composition: ASC1: *P. pentosaceus* (S4XNM) and *S. xylosus* (S8HS); ASC2: *P. pentosaceus* (S8QM) and *S. xylosus* (S8HS); ASC3: *P. pentosaceus* (S4XNM), *P. pentosaceus* (S8QM) and *S. xylosus* (S8HS); ASC4: *L. sakei* (S29ZEM) and S. *xylosus* (S8HS); ASC4: *L. sakei* (S29BM) and *S. xylosus* (S8HS); ASC6: *L. sakei* (S29ZEM), L. sakei (S29BM) and *S. xylosus* (S8HS); ASC7: *L. sakei* (S29ZEM), *P. pentosaceus* (S4XNM) and *S. xylosus* (S8HS).

### 5.3.7. Effect of autochthonous starter cultures on volatilome profiles

Dry sausages fermented with the seven ASC and control showed several differences in their volatilome profiles both qualitatively and quantitatively (Supplementary Table 5). Sausages obtained produced by *L. sakei* strains harbored specific volatile organic compounds (VOCs) in concentration similar to the control one, *i.e.*, hexanal, isopentyl alcohol, 2-methyl-3-octanone, 1-hexanol, acetic acid, 1-octanol, butyrolactone and hexanoic acid. All such molecules showed a lower concentration ($P < 0.05$) in the samples produced with ASC composed by *P. pentosaceus* strains except for hexanoic acid.

Sausages only inoculated with *P. pentosaceus* strains did not contain detectable butanoic acid ethyl ester, butanoic acid methyl ester, hexanoic acid and octanoic acid (Supplementary Table 5, $P<0.05$). In contrast, acetoin and diacetyl were at higher concentrations in ASC1, 2 and 3 sausages (all containing *P. pentosaceus* strains) and in the control.

Some ASC sausages were also characterized by qualitative and quantitative differences in their volatile profiles. For example sausages produced with ASC 1 showed the highest concentration of 2-methyl-butanal, 3-methyl-butanal, hexanal, 2-methyl-1-propanol, 1-butanol, styrene, 6-methyl-5-heptene-2-one, 2-ethyl-1-hexanol, dimethyl sulfone and absence of ethyl isovalerate. On the other hand, samples from ASC 5 were characterized by a high concentrations of allyl methyl sulphide, methyl octanoate, nonanal, ethyl decanoate, butanoic acid methyl ester, butanoic acid ethyl ester, ethyl 2-methylbutanoate, ethyl isovalerate, methyl caproate and absence of 1-octen-3-ol (Supplementary Table 5, $P<0.05$). No significant differences between the ASC samples and the control was observed for the following compounds: methyl isobutyrate, 2-butoxy-ethanol, isobutyric acid, butanoic acid and 3-methyl-butanoic acid. It also important take into account that there were important differences in CNS counts between different ASCs. S. xylosus population showed the highest abundances in ASC1, 2 and 3 as compared to ASC4, 5, 6 and 7 and the lowest in the control. These differentia could have a significative impact on the VOCs composition.

The PCA-biplot (Figure 4), in which only the top 9 contributing volatile compounds are shown, explains properly sample grouping based on their volatile composition at the end of the fermentation. The volatile profiles of the three sausages inoculated with ASC composed of *P. pentosaceus* strains (ASC 1, 2, 3) clustered together, while those inoculated with ASC 5 and 6, composed of *L. sakei* S29BM and combination of two *L. sakei* strains (S29ZEM and S29BM) respectively, clustered together. Between these two groups, we found that of ASC 7, which was composed of *P. pentosaceus* S4XNM and *L. sakei* S29ZEM, and the ASC 4 composed of *L. sakei* S29ZEM (Figure 4). The samples inoculated with ASC 5 were the most different if compared to the control.

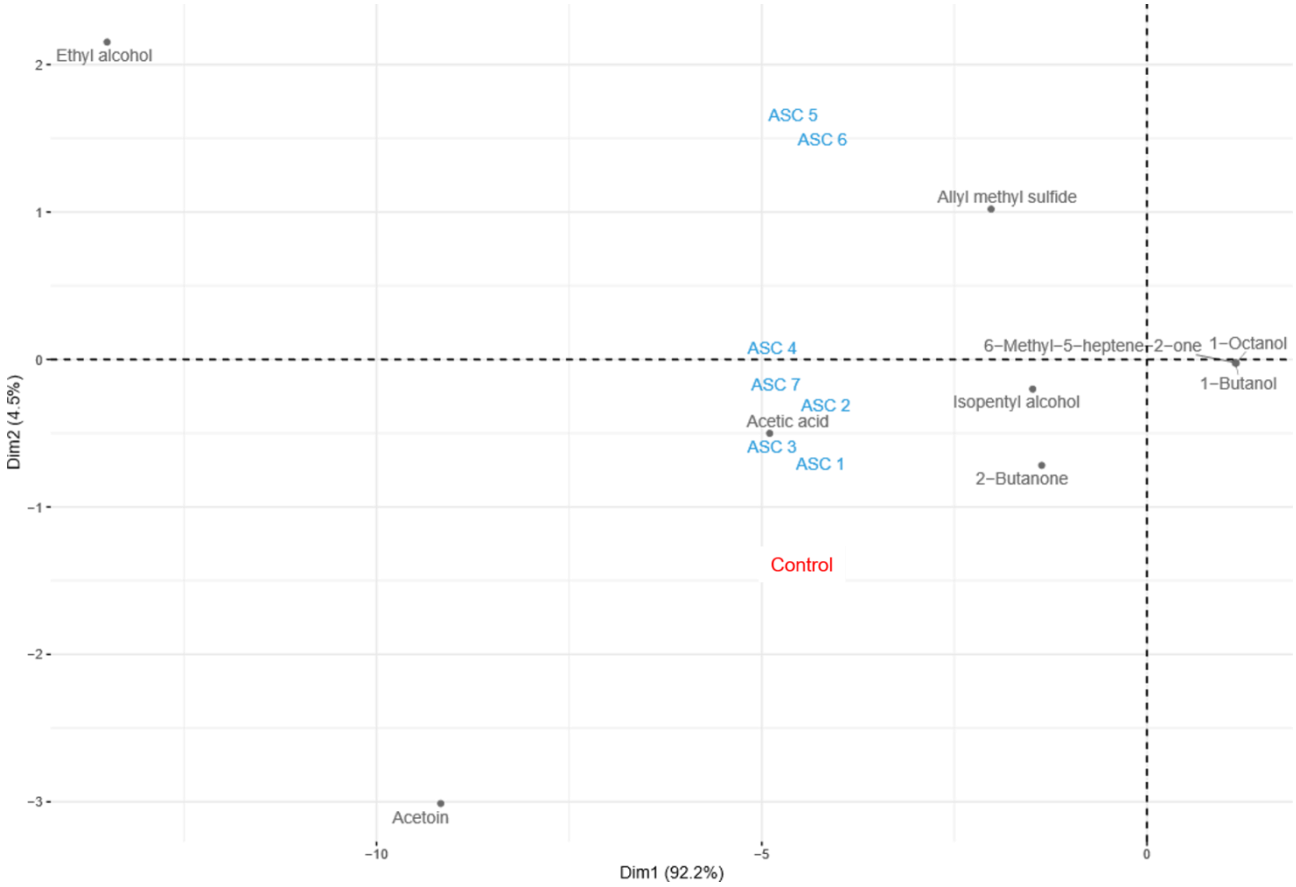


Figure 4. Principal component analysis (PCA) Biplot showing sausages samples grouping according to volatilome composition. Only the most nine significant VOCs are show.

### 5.3.8. Effect on sensory attributes of inoculated sausages

The sausages produced with the ASC were evaluated by 20 consumers and compared with control sausages. Overall, the liking test suggested a higher consumer preference for sausages manufactured with ASC as compared to the control (Figure 5).

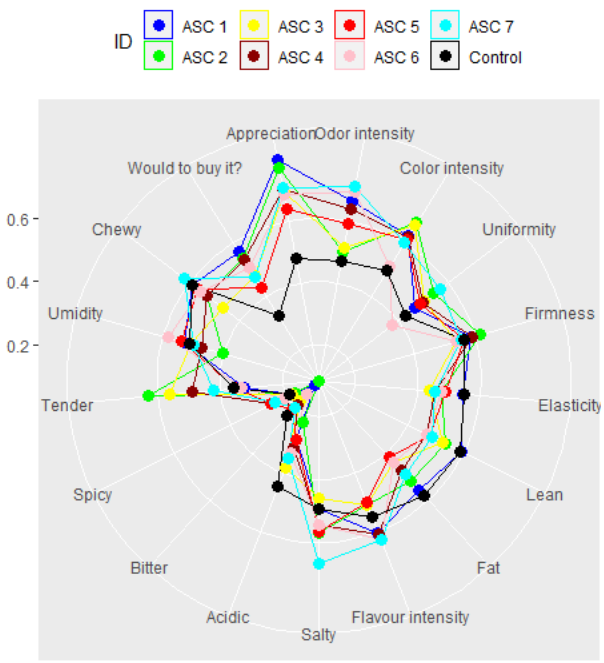


Figure 5. Radar graphs displaying the liking of appearance, odor, taste, flavor, and texture and overall liking expressed by consumers for the sausages made by Standard starter cultures (Control) and inoculated fermentation

In particular, sausages produced with ASC 1 (*P. pentosaceus* S4XNM) and ASC 2 (*P. pentosaceus* S8QM) were considered the best in terms of acceptability (P<0.05). Comparing all attributes of sausages produced with ASC against the control, it was found that they were less bitter, acidic, and that fat and lean parts were less visible, resulting in a more uniform aspect. All ASC inoculated samples also exhibited the highest scores for odour and colour intensity (P<0.05). Samples produced with ASC 7 (*P. pentosaceus* and *L. sakei)* were evaluated as more balanced for most of the investigated attributes, with highest salt (P<0.05), flavour and odour intensity. Samples produced with ASC 5 showed a medium score for all descriptors and obtained the lowest score for general appreciation (P<0.05). All ASC inoculated samples obtained a higher score than the control for the question "Would you buy it?", in particular sausages produced with ASC 1 (P<0.05). It should be pointed out that sausages inoculated with ASC 1, ASC 2, ASC 3 composed by *P. pentosaceus* showed the highest scores for colour intensity and general appreciation (P<0.05) if compared with samples with *L. sakei* strains that were perceived as the most acidic (P<0.05).

### 5.4. Discussion

Fermented sausages are the result of complex microbiological activities, firstly the acidification of the meat batter (Belleggia *et al*., 2020b), followed by several biochemical reactions that allow the formation of typical aromatic compounds. Regarding naturally fermented sausages, we observed that samples belonging to the February batch showed a lower pH value at the end of fermentation as compared with the other two batches. This is due to the strong acidification capability of the LAB strains that composed the microbiota of this batch. On the opposite, LAB strains that characterized the microbiota of the March batch decreased the pH value by less than one unit (0.31). This was probably due to the weak acidification capability of the strains present in this batch. Microbiota composition of the naturally fermented sausages showed differences for the three batches. Studying the product, we focused not only on *L. sakei* strains, but also on *P. pentosaceus* strains. The latter is known for its high acidification capability, that could be a positive quality for use as a starter culture in fermented meat for high acidity products, besides a significant antioxidant and flavour formation potential (Chen et al., 2015a, 2015b).

February samples showed a high predominance of *P. pentosaceus*, while May samples were dominated by *L. sakei*. In fact, most of *P. pentosaceus* isolates selected for meat inoculation belonged to the February batch (Table 2). March was the only batch were *L. curvatus*, *Loi. coryniformis* and *Lpb. plantarum* were isolated. These results also highlighted the highest acidification capability of *P. pentosaceus* strains which were dominant in the most acidic batch (February). More acidic fermented meat products, generally from Northern Europe, show a higher prevalence of *P. pentosaceus* in their LAB communities (Van Reckem et al., 2019). On the other hand, the lower acidification rate of *L. sakei* strains could explain why the March batch (that was characterized by a high presence of *L. sakei* strains) was the less acidic final product. In fact, the two selected *L. sakei* strains belonged to the March batch and showed a lower acidification capability as compared to the *P. pentosaceus* isolates from the February batch (Table 2). We observed that the acidification rate of different isolates was species-dependent, in fact isolates belonging to February batches, which were characterized by high presence of *P. pentosaceus*, showed a high acidification rate at 20°C. The use of these strains for meat inoculation (ASC 1, 2 and 3) yielded a rapid decrease in pH value in the first days of fermentation, the same trend was also observed for control sausages although inoculated with *L. sakei*. It has already been reported that *P. pentosaceus* was preferred to other LAB species for their acidification properties to obtain adequate sausage fermentation. Species belonging to *Pediococcus* genus are actually used in the food industry as protective cultures against common food spoilage bacteria (Porto *et al*., 2017) and as starter cultures for high acidity sausages (Chen *et al*., 2015b, 2015a). *P. pentosaceus* is also important for its antioxidant ability (Kim *et al*., 2019), its abilities to generate odour precursors that contribute to formation of specific flavour (Sun *et al*., 2019) and to prevent excessive lipid oxidation and subsequent off-flavour production (Chen *et al*., 2015a, 2015b).

For the CNS group, we did not observe a constant increase in their populations during fermentation, and their abundance was always lower to that of LAB as previously reported for other naturally fermented sausages (Cardinali *et al*., 2018; Ferrocino *et al*., 2018; Rantsiou *et al.*, 2005b). *S. xylosus* was the most dominant species

in all batches, while other species were randomly isolated. The dominance of *L. sakei* and *S. xylosus* in all batches of spontaneously fermented sausages was in accordance with literature data (Eisenbach *et al*., 2019; Iacumin *et al*., 2020; Pini *et al*., 2020; Van Reckem *et al.*, 2019).

NaCl tolerance is one of the criteria used to select autochthonous starters since it is a major factor affecting bacterial growth during fermentation (Cruxen *et al*., 2019; Laslo *et al*., 2020). In fact, salt is generally used at different concentrations in processed meat to develop taste and extend shelf life. For most isolates, an increase in salt concentration reduced bacterial growth as previously reported in the literature (Aina, 2017). It is known that the ability of microorganisms to grow under different conditions is species-dependent (Cruxen *et al*., 2019) and also strain-dependent (Casaburi *et al*., 2011; Ercolini *et al*., 2010), as observed in the present study.

Concerning the effect of temperature on growth, it can be said that almost all isolates grew at 30°C, while the number of LAB isolates able to grow decreased with a decrease in temperature. CNS isolates displayed a higher proportion of isolates able to grow at low temperature (10°C) compared to high temperatures, although with slower growth. This is an unexpected result, because it is known that the optimum growth temperature of *S. xylosus* is 30°C (Essid *et al*., 2007), although it is able to grow well at temperatures normally used for meat fermentation (10 and 20°C) (Essid *et al*., 2007). All the results described above were important to select strains able to grow well at the salt concentration (2-3 %) and temperature range (20-10°C) encountered during Salame Piemonte production. Finally, the selected CNS strains showed proteolytic, lipolytic and nitrate reductase activity on the assayed agar medium, which was important given the beneficial support of these activities to texture, flavor and color development (Laslo *et al*., 2020).

Concerning safety aspects, before using any strain for pilot-scale production and future industrial applications, the selected strains were evaluated based on biogenic amine gene detection and antibiotic resistance (MIC determination) allowing to discard LAB isolates harboring decarboxylase genes in some cases and intermediate resistance to antibiotics.

The fermentation process by using our ASC satisfied the standard requirements of Salame Piemonte since the directive reported in the disciplinary (Official Gazette of the Italian Republic, nr 184, 10 August 2015) requires the final pH value to be equal or higher than 5.2. In addition, a correct acidification process favors inhibition of *Listeria* spp. (Zaiko *et al*., 2020).

The sensorial characteristics of the final products were related with the different strains of lactic acid bacteria in the ASC formulation, as well as inoculated *S. xylosus* and *P. nalgiovense* strain activity. The difference in the volatilome profiles can be related to the complex synergic interaction of microbiota activities. VOCs are generally the result of proteolysis, lipolysis and carbohydrate metabolism, beside wine and spices (Chen *et al*., 2015a, 2015b). Glucose and carbohydrate pathways were found to be one of the main precursors of many VOCs such as acetate, acetoin, diacetyl, acetic acid and isobutyric acid (Casaburi *et al*., 2015; Ferrocino *et al*., 2018).

In general, we observed that the sausages most rich in aromatic components were those obtained with ASC 1 (*P. pentosaceus* S4XNM and *S. xylosus* S8HS) and 5 (*L. sakei* S29BM and *S. xylosus* S8HS), while for ASC 7 (*P. pentosaceus* S4XNM, *L. sakei* S29ZEM and *S. xylosus* S8HS), its volatilome profile was more similar to

that of ASC 4 (*L. sakei* S29ZEM and *S. xylosus* S8HS). In particular, *L. sakei* S29BM had a very significant impact on the sausage volatilome. The volatilome of sausages produced with ASC 6 (*L. sakei* S29BM and S29ZEM) was more similar to that of ASC 5 (*L. sakei* S29BM) than to that of ASC 4 (*L. sakei* S29ZEM) (Figure 4).

The highest concentrations of diacetyl and acetoin were found in samples in which *P. pentosaceus* strains were used to drive the fermentation (ASC 1). These compounds are products of the carbohydrate catabolism of LAB and staphylococci and are associated with dairy odours found mostly in fresh meat (Montel *et al*., 1998). It is known that *Pediococcus* genera produce more acetoin than *L. sakei*, in particular *P. pentosaceus* which is often associated with acetoin and diacetyl production (Sunesen *et al*., 2004). Samples inoculated with *P. pentosaceus* strains, that were also characterized with the highest *S. xylosus* populations, also showed a high concentration of alcohol compounds, *i.e*. ethanol (ethyl alcohol), isopentyl alcohol, 1-hexanol and 1-octanol. 1-octanol gives specific odour attributes referred to as waxy, green, citrus and floral with a sweet and coconut nuance (Casaburi *et al*., 2015), while the attributes related to 1-hexanol are cheese, oxidized fat, rancid and humidity (Perea-Sanz *et al*., 2018). Other works reported the capability of *P. pentosaceus* to give aromatic characteristics to the final product, finding the highest aldehyde, alcohol and acid contents in samples inoculated with *P. pentosaceus* (Chen *et al*., 2015b; Cruxen *et al*., 2019).

The content of hexanoic acid and octanoic acid which originate from the oxidation of corresponding alcohols (Hu *et al*., 2019), were both higher in sausages made with *L. sakei* strains (ASC 4, 5, 6) as compared with *P. pentosaceus* inoculated sausages (ASC 1, 2, 3). Although in other cases a low concentration of acetic acid has been reported as preferred from a sensory point of view (Iacumin *et al*., 2020), in our study, consumers preferred the sausages with the highest concentration. Indeed, the liking test showed that consumers preferred sausages produced using ASC 1 (*P. pentosaceus* S4XNM) which exhibited the highest concentration of acetic acid. The preference for this sausage by the consumers was probably due to the combination of low saltiness perception ($P < 0.05$), high odour intensity ($P < 0.05$) and texture characteristics (tenderness, $P < 0.05$). Consumer preference was also related to *S. xylosus* populations. Indeed, *S. xylosus* counts were 2-3 $\log_{10}$ higher in ASC1, 2 and 3 sausages (containing *P. pentosaceus)* than in the other ASC and control sausages (containing *L. sakei)* . Given the role of CNS in meat fermentation (color, lipolysis, proteolysis) (Iacumin *et al.*, 2006), it is likely that *S. xylosus* contribution to ASC1, 2 and 3 sausage organoleptic properties was higher, which together with *P. pentosaceus* metabolic activities yielded final products which were preferred by consumers.

Based on rep-PCR fingerprinting of isolates collected during fermentation and metataxonomic analysis, we confirmed that the inoculated strains were dominant from the beginning and until the end of the fermentation process. Other subdominant species were identified through metataxonomic analysis, but their relative abundance was low so we can suppose that their contribution to the final sensory properties was not major. Therefore, we can affirm that the selected strains significantly contributed to the product final characteristics. We observed that the inoculated strains became rapidly dominant at the beginning of fermentation and played a major role in the fermentation process that transforms the meat's sensory profile into the typical organoleptic properties of fermented sausages.

### 5.5. References

References of this chapter are integrated into the overall reference section (Chapter 7).

### 5.6. Data availability

Sequences have been uploaded to the National Center for Biotechnology Information Sequence Read Archive (Bioproject ID PRJNA669431).

# Chapter 6 – General conclusions

Artisanal fermented products are characterized by a high level of microbial variability (Iacumin *et al*., 2020) that could increase the risk of food spoilage. Only an accurate management of microbial resources by means of specific (autochthonous) starter cultures allows to reduce product variability and to ensure a good quality level. Undoubtedly, the addition of starter culture strains in the meat batter for the production of sausages makes the fermentation process less prone to modifications, which are responsible for products with altered 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 especially in 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 products with homogeneous organoleptic characteristics.

To select a new starter culture, it is necessary to study the dynamics and diversity of the autochthonous microbiota of the fermented product. Our study demonstrated that during a spontaneous fermentation process, different microorganism could take part to the biochemical transformations allowing the formation of specific organoleptic characteristics. It is fundamental to take into account that different microbiota structure implies the formation of specific VOCs that influence the final sensory properties. In fact, in this study, we observed a clear distribution of the main LAB species (*L. curvatus, L. sakei, P. pentosaceus*) in the three studied batches (February, March; May) demonstrating the interconnection between the initial microbiota of the meat and that of the product itself. For example, at the end of the spontaneous fermentation process, samples obtained from March showed the presence of unpleasant metabolites correlated to the presence of *L. curvatus*. In addition, the different metabolomic characteristics of the batches were not only linked to the species level, but also to the strain-level diversity, in particular in the case of *L. sakei*. The metagenomics data indicated as more suitable for starter cultures formulation strains belong to *L. sakei* and *P. pentosaceus* species. Obtaining information on single strains, but also on interaction between strains belonging to the same species, helps to better understand this complex microbial ecosystem and to select new starter cultures for this type of fermented foods.

The main risk linked to the use of commercial starter cultures is the loss of the traditional product's characteristics and to avoid this, it requires studying the microbiota of naturally fermented sausages as a source of autochthonous strains. Testing different strain combinations is fundamental to observe how they impact fermentation and to select the ones performing the best in the required conditions. In our case, sensory properties were found to be more influenced by LAB activity at the strain level. The obtained VOC data underlined the ability of *P. pentosaceus* strains to confer desirable characteristic (flavour, odour and colour intensity) as highlighted by the liking test. The results revealed that *P. pentosaceus* together with *S. xylosus* could be appropriate for use as alternative meat starter culture for *Salame Piemonte* production, as already reported for other similar products in which a high level of acidity is desirable (Chen *et al*., 2015b; Porto *et*

*al*., 2017). In details, the best solution could be the use of a mixed culture of *P. pentosauces* S4XNM, to obtain desirable organoleptic characteristics.

However, we should not forget the importance of mycobiota diversity and dynamics that colonize the surface of traditionally fermented meat products, as they have an important role in their sensory properties. This study highlighted the substantial fungal diversity in both meat and casing surface samples throughout maturation despite the fact that the product was deliberately inoculated with a *P. nalgiovense* commercial adjunct culture. It also showed the important contribution of autochthonous fungi in the microbial ecology of this product, which together with the bacterial microbiota and Salame Piemonte process specifications, led to the typical organoleptic properties of this product. Future in-depth studies on the contribution of this mycobiota on the product volatilome profile using metagenomics or metatranscriptomic approaches could be interesting in order to select fungal cultures to be used with the bacteria starter cultures.

The present study has shown that the application of modern molecular methods, such as metataxonomics and metagenomics, in fermented sausages allows a better in-depth understanding of the microbial ecology and functions and, at the same time, a better comprehension of the interactions of the starter cultures with the meat microbiota during sausage production. It is only after a thorough knowledge of the mechanisms behind the meat fermentation process that it will be possible to efficiently control and modulate the meat microbiota to obtain products with desired organoleptic properties.

# Chapter 7 - References


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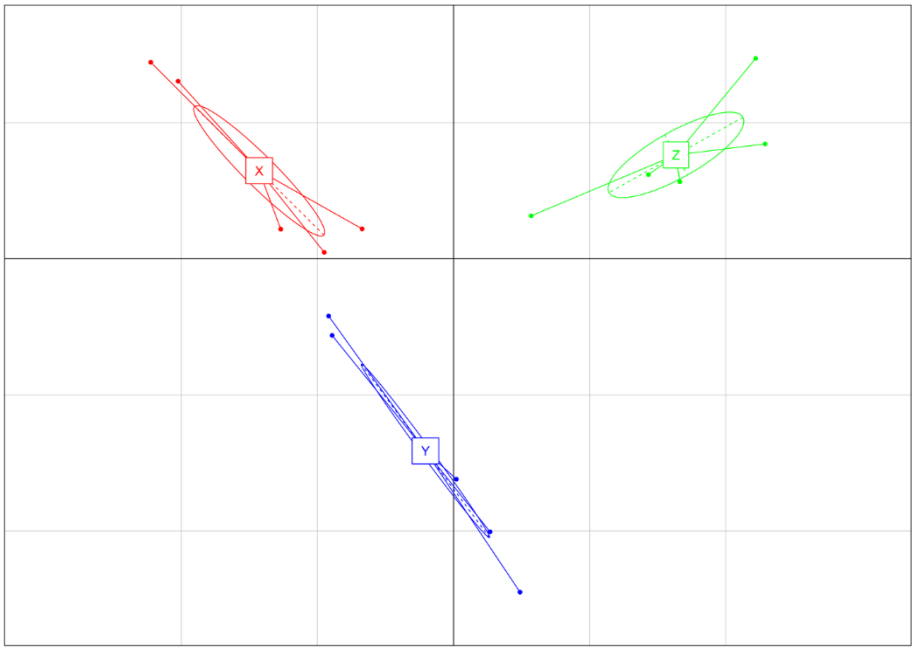
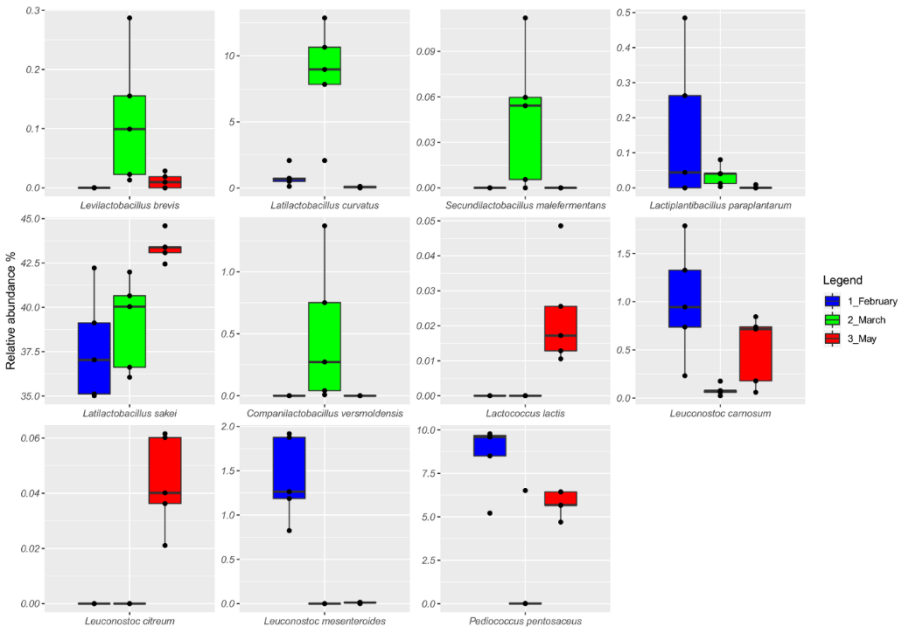
# Chapter 8 - Annex

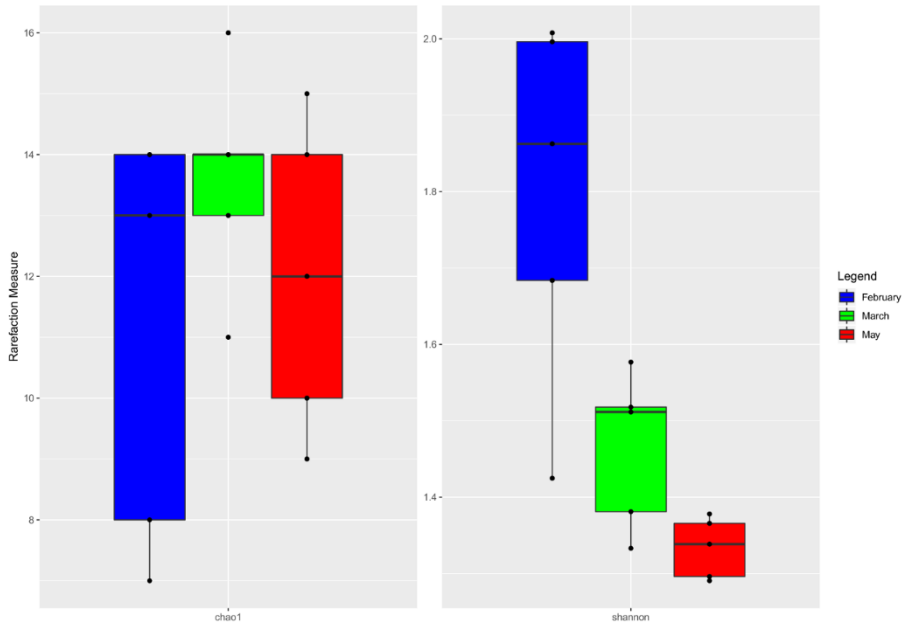
## 8.1. Supplementary Materials Chapter 3



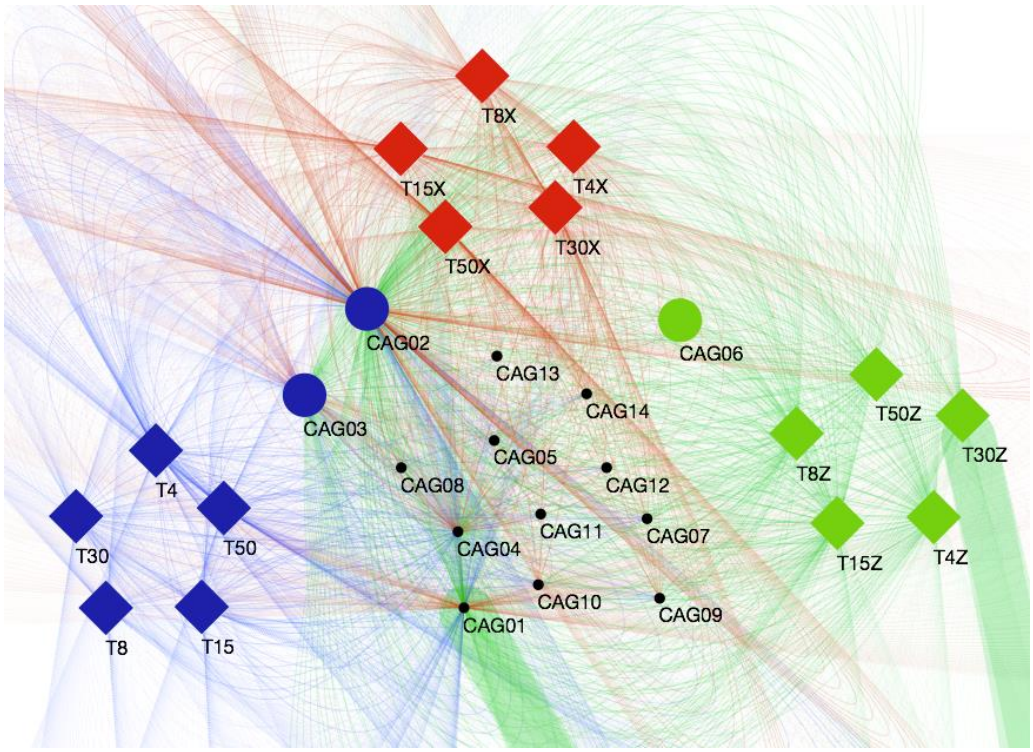

**Supplementary Figure 1:** PCA based on the specific minor microbiota that drove the cluster separation of the three batches (February in blue, March in green, May in red). The first component (horizontal) accounts for 33.92% of the variance and the second component (vertical) accounts for 24.01%.



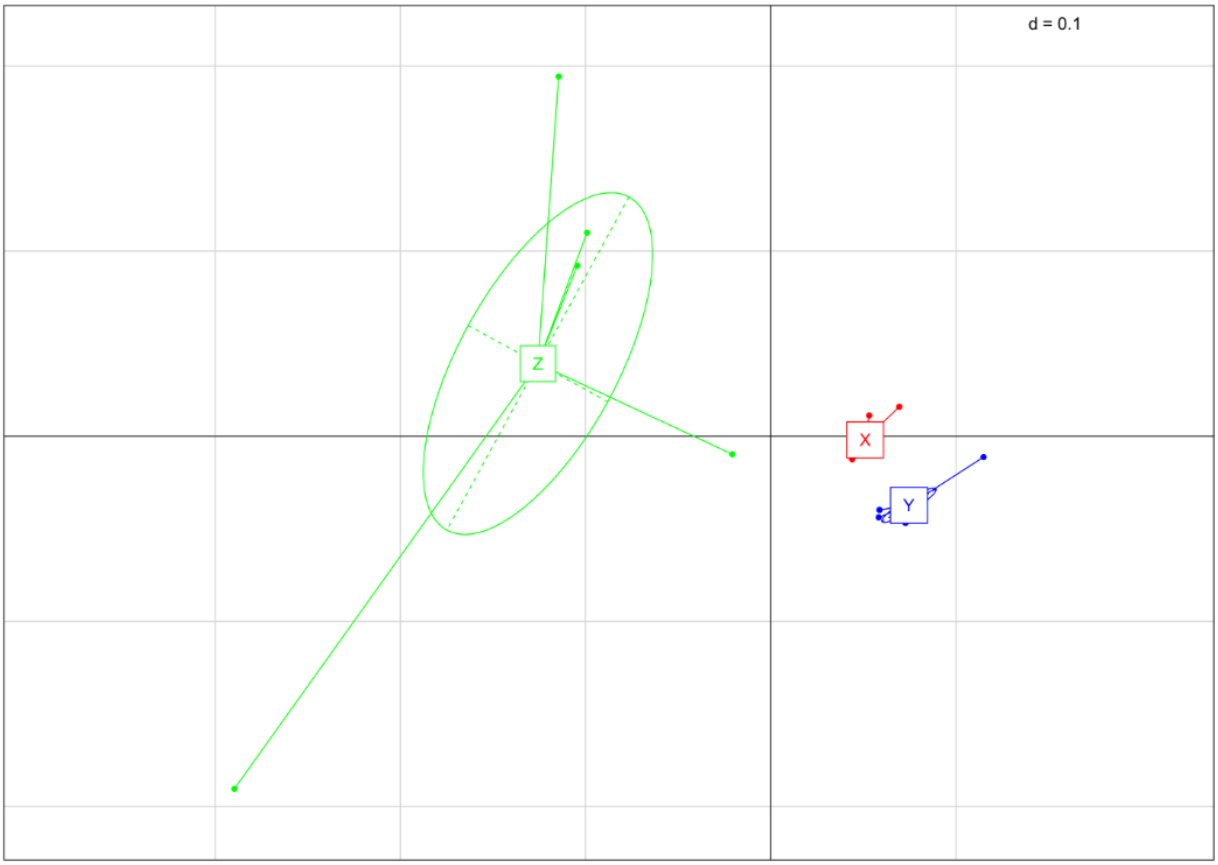

**Supplementary Figure 2**: Boxplots showing the relative abundance of OTUs differentially abundant based on Wilcoxon matched pairs test (FDR < 0.05)in sausages samples from February (blue bars), March (green bars) and May (red bar).



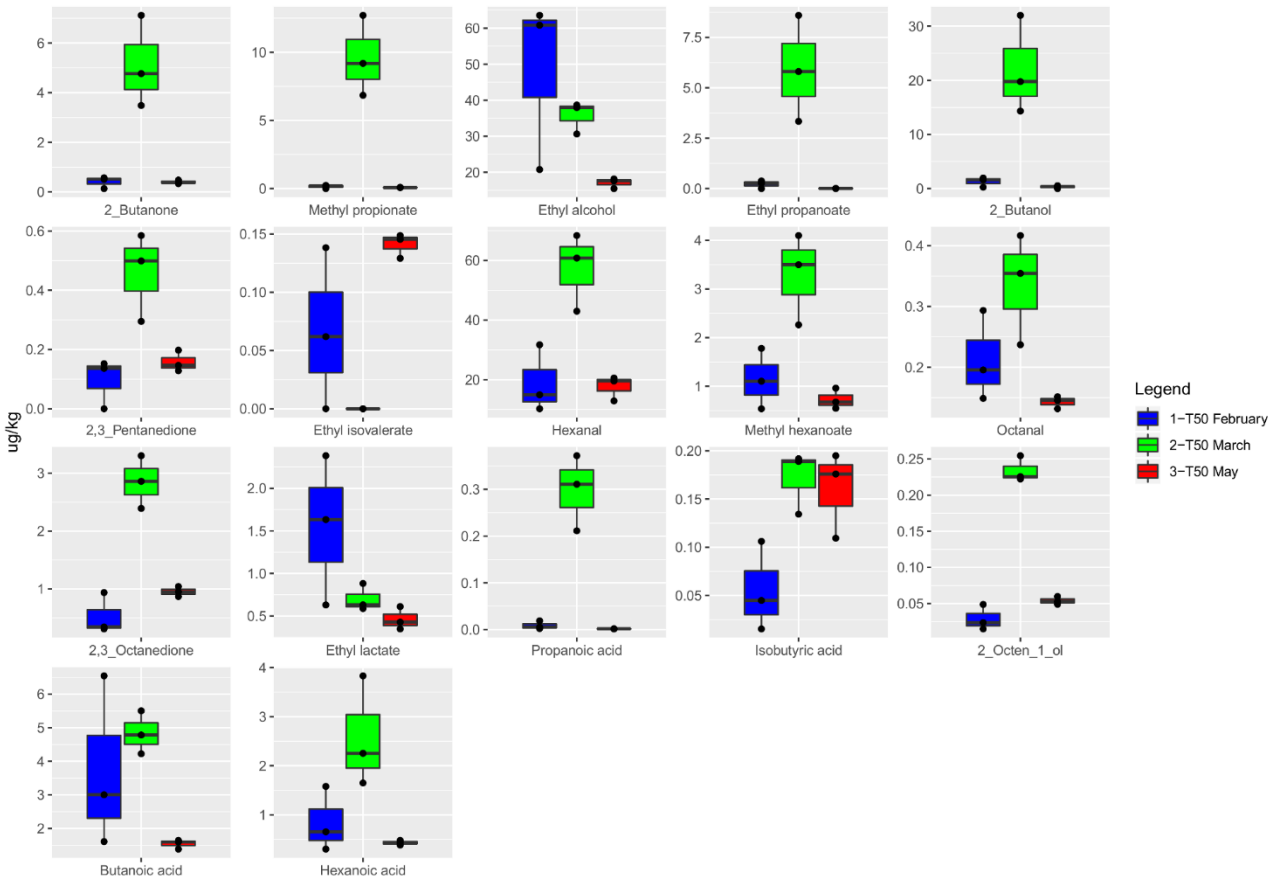

**Supplementary Figure 3**: Boxplot explaining the alpha diversity (Chao and Shannon index) between the three batches.



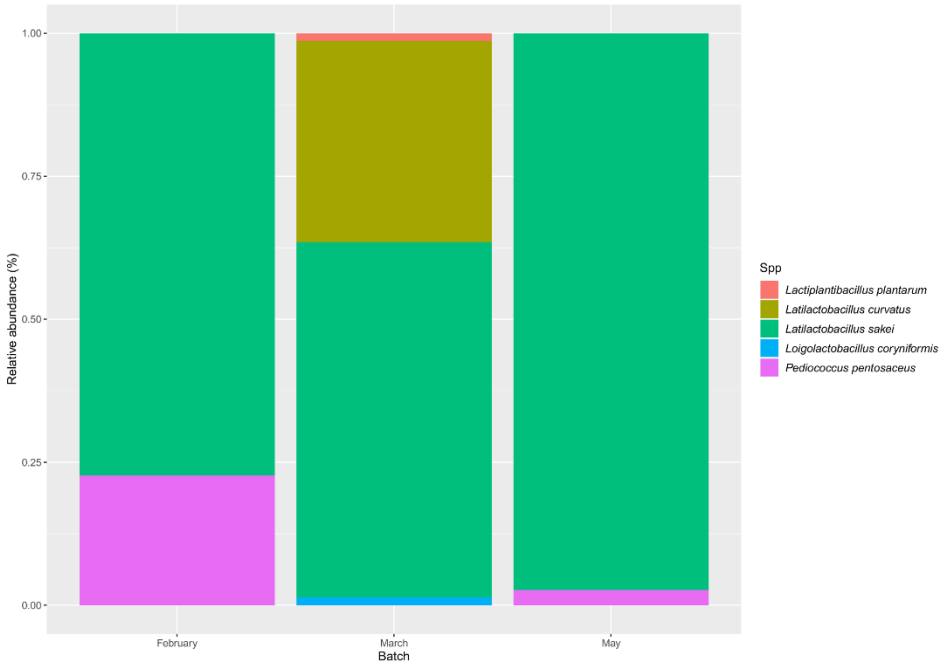

**Supplementary Figure 4**: Spearman correlation between Co-Abundance Gene Groups (CAGs) and sausages from the three different batches: February samples in blue, March samples in green and May samples in red.



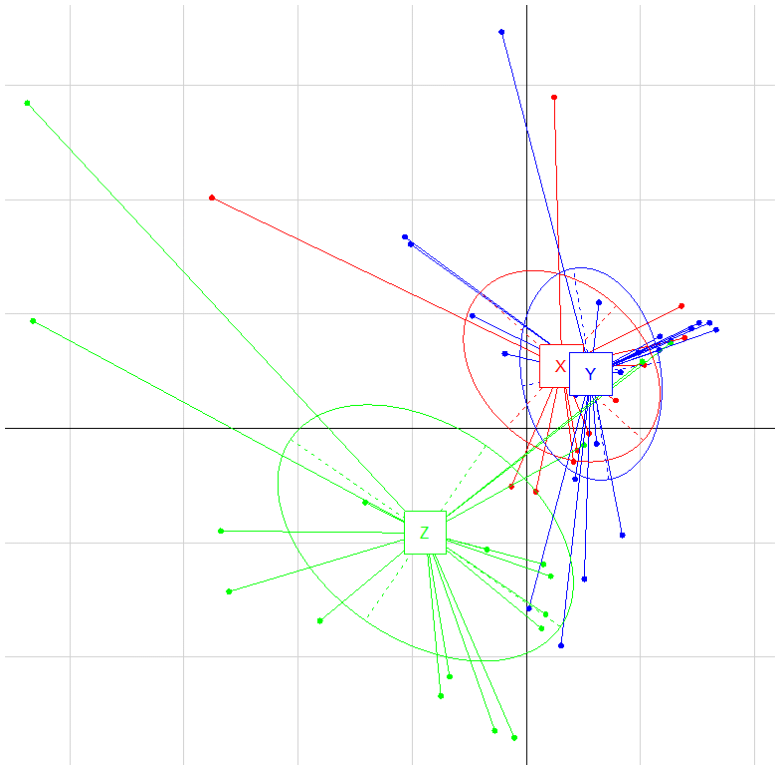

**Supplementary Figure 5:** Principal coordinates analysis based on *Latilactobacillus sakei* pangenome (February in blue, March in green, May in red).




**Supplementary Figure 6**: Boxplot of the distribution of volatile organic compounds (VOCs) at the end of fermentation differentially abundant based on Wilcoxon matched pairs test (FDR < 0.05) in sausages samples from February (blue bars), March (green bars) and May (red bar).




**Supplementary Figure 7**: Overall relative abundance (%) of Lactic Acid Bacteria (LAB) species in three different batches by culture dependent approach.




**Supplementary Figure 8**: PLS-DA models based on *Latilactobacillus sakei* REP fingerprints of the three batches (February in blue, March in green and May in red).

**Supplementary Table 1:** Volatile compounds (µg/kg) determined on sausages samples during ripening. Lower Case Letters in the same row indicate significant differences ($P < 0.05$) between the different time in the same batch; Capital Letters in the same row indicate significant differences ($P < 0.05$) between same time in different batches.

| | | **February** | | | | |
|---|---|---|---|---|---|---|
| **Class** | **Volatile compounds (ppb) (µg/kg)** | **T4** | **T8** | **T15** | **T30** | **T50** |
| Esters | Ethyl propanoate | 0.00 ± 0.00aA | 0.00 ± 0.00aA | 0.00 ± 0.00aA | 0.00 ± 0.00aA | 0.22 ± 0.20bA |
| | Ethyl isovalerate | 0.00 ± 0.00aA | 0.00 ± 0.00aA | 0.00 ± 0.00aA | 0.00 ± 0.00aA | 0.07 ± 0.07bAB |
| | Ethyl lactate | 1.19 ± 0.05aA | 1.62 ± 0.60aB | 0.79 ± 0.09aA | 1.66 ± 0.24aB | 1.59 ± 0.88aB |
| | Methyl hexanoate | 1.61 ± 0.64aA | 1.33 ± 0.47aB | 2.23 ± 0.41abB | 2.88 ± 0.97bA | 1.14 ± 0.62aA |
| | Butanoic acid, ethyl ester | 1.15 ± 0.39aB | 1.11 ± 0.38aB | 0.73 ± 0.06aA | 1.23 ± 0.31aB | 1.13 ± 0.74aA |
| | Methyl butyrate | 0.02 ± 0.00aB | 0.02 ± 0.01aA | 0.01 ± 0.00aA | 0.03 ± 0.01aA | 0.03 ± 0.02aA |
| | Methyl propionate | 0.00 ± 0.00aA | 0.00 ± 0.00aA | 0.00 ± 0.00aA | 0.11 ± 0.06abA | 0.15 ± 0.13bA |
| Ethers | 2-Butoxyethanol | 0.28 ± 0.03aAB | 0.29 ± 0.08aAB | 0.52 ± 0.12bA | 0.52 ± 0.05bA | 0.25 ± 0.12aA |
| Alcohols | Ethyl alcohol | 53.88 ± 8.53aB | 45.86 ± 5.29aC | 61.78 ± 5.08aB | 48.93 ± 4.85aB | 48.39 ± 23.95aB |
| | 2-Butanol | 0.09 ± 0.00aA | 0.13 ± 0.06aA | 0.11 ± 0.01aA | 0.29 ± 0.07aA | 1.28 ± 0.90bA |
| | Isopentyl alcohol | 5.47 ± 1.06aB | 3.90 ± 0.48aB | 4.36 ± 0.12aA | 4.10 ± 0.50aAB | 3.68 ± 2.39aA |
| | 1-Hexanol | 8.84 ± 8.95aA | 4.66 ± 2.76aA | 51.23 ± 12.75bB | 4.45 ± 2.42aA | 2.57 ± 2.27aA |
| | 2-Octen-1-ol | 0.23 ± 0.12bA | 0.07 ± 0.02aA | 0.42 ± 0.07cB | 0.26 ± 0.10bB | 0.03 ± 0.02aA |
| Aldehydes | Hexanal | 49.42 ± 17.58abA | 26.73 ± 4.52aB | 104.69 ± 29.90cB | 82.44 ± 31.65bcB | 18.98 ± 11.28aA |
| | Nonanal | 0.74 ± 0.44aAB | 0.41 ± 0.10aA | 4.51 ± 1.56bB | 1.34 ± 0.83aA | 0.52 ± 0.44aA |
| | Heptanal | 1.21 ± 0.61aA | 0.60 ± 0.15aB | 6.33 ± 2.00bB | 1.92 ± 1.04aB | 0.24 ± 0.26aA |
| | Octanal | 0.58 ± 0.34aA | 0.25 ± 0.06aA | 3.43 ± 1.42bB | 1.00 ± 0.64aA | 0.21 ± 0.07aAB |
| | Pentanal | 5.83 ± 1.65aA | 3.14 ± 0.29aA | 21.49 ± 5.59cB | 11.78 ± 4.13bB | 2.16 ± 1.12aA |
| Ketones | Acetoin | 0.13 ± 0.12abAB | 0.01 ± 0.00aA | 0.17 ± 0.07bB | 0.027 ± 0.01aA | 0.01 ± 0.01aA |
| | 2-Heptanone | 0.28 ± 0.37aA | 0.22 ± 0.30aA | 0.20 ± 0.05aB | 0.09 ± 0.06aA | 0.02 ± 0.02aA |
| | 2,3-Octanedione | 1.92 ± 0.83bcA | 0.77 ± 0.22abA | 2.39 ± 0.22cB | 2.43 ± 1.08cA | 0.53 ± 0.35aA |
| | 2,3-Pentanedione | 0.41 ± 0.1bcB | 0.12 ± 0.013aA | 0.20 ± 0.04aA | 0.54 ± 0.10bA | 0.10 ± 0.08aA |
| | 2-Butanone | 0.52 ± 0.26aAB | 0.41 ± 0.11aAB | 0.58 ± 0.10aA | 0.55 ± 0.25aA | 0.41 ± 0.24aA |
| Acids | Acetic acid | 8.31 ± 3.84aA | 13.82 ± 1.21abA | 14.92 ± 6.38abA | 26.81 ± 5.22abB | 32.64 ± 24.72bA |
| | Propanoic acid | 0.00 ± 0.00aA | 0.00 ± 0.00aB | 0.01 ± 0.00aA | 0.01 ± 0.00aA | 0.01 ± 0.01aA |
| | Isobutyric acid | 0.00 ± 0.00aA | 0.00 ± 0.00aA | 0.00 ± 0.00aA | 0.00 ± 0.00aB | 0.06 ± 0.05bA |
| | Butanoic acid | 3.14 ± 1.76aA | 3.05 ± 0.41aA | 4.81 ± 1.08aA | 4.11 ± 0.20aAB | 3.72 ± 2.54aAB |
| | Hexanoic acid | 2.45 ± 2.34aA | 1.57 ± 0.68aA | 8.69 ± 2.26bB | 2.94 ± 0.84aB | 0.84 ± 0.66aA |
| Sulphur compounds | Dimethyl sulfone | 0.10 ± 0.08aA | 0.10 ± 0.02aB | 0.09 ± 0.06aA | 0.15 ± 0.03aB | 0.26 ± 0.19aA |
| Hydrocarbon | 2-Octene | 0.01 ± 0.08abA | 0.06 ± 0.09aA | 0.17 ± 0.01bB | 0.00 ± 0.00aA | 0.00 ± 0.00aA |
| | | **March** | | | | |
| Class | Volatile compounds (ppb) (µg/kg) | **T4** | **T8** | **T15** | **T30** | **T50** |
| Esters | Ethyl propanoate | 0.00 ± 0.00aA | 0.01 ± 0.02aA | 0.10 ± 0.00aA | 0.87 ± 0.42aB | 5.91 ± 0.00bB |
| | Ethyl isovalerate | 0.00 ± 0.00aA | 0.00 ± 0.00aA | 0.00 ± 0.00aA | 0.05 ± 0.01bA | 0.00 ± 0.00aA |
| | Ethyl lactate | 0.75 ± 0.14bA | 0.71 ± 0.06bA | 1.42 ± 0.14cB | 0.14 ± 0.24aA | 0.70 ± 0.14bAB |
| | Methyl hexanoate | 0.97 ± 0.19aA | 0.71 ± 0.08aA | 1.52 ± 0.19aA | 1.53 ± 1.02aA | 3.29 ± 0.19bB |

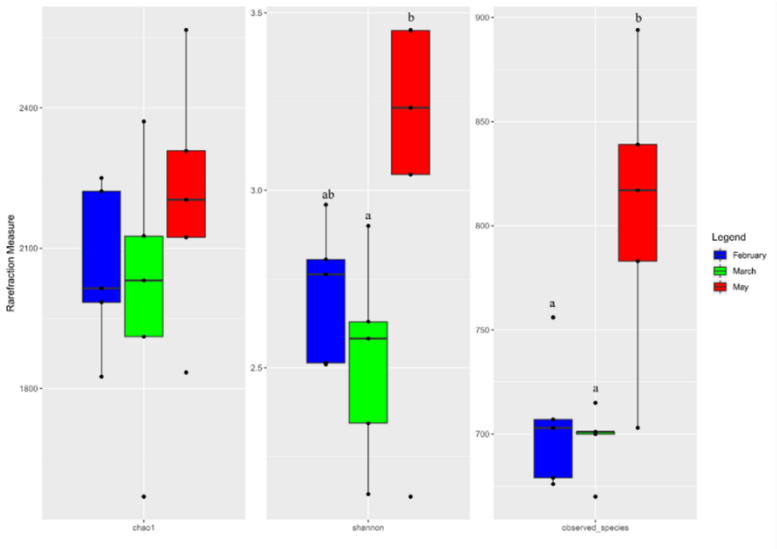
| | | | | | | |
|---|---|---|---|---|---|---|
| | Butanoic acid, ethyl ester | 0.52 ± 0.14abA | 0.53 ± 0.03abA | 0.94 ± 0.16bA | 0.32 ± 0.18aA | 0.96 ± 0.14bA |
| | Methyl butyrate | 0.01 ± 0.00aA | 0.02 ± 0.00aA | 0.04 ± 0.00abB | 0.03 ± 0.01aA | 0.06 ± 0.00bA |
| | Methyl propionate | 0.05 ± 0.03aB | 0.06 ± 0.07aA | 0.51 ± 0.03aB | 1.66 ± 0.31aB | 9.58 ± 0.03bB |
| Ethers | 2-Butoxyethanol | 0.22 ± 0.08aA | 0.16 ± 0.03aA | 0.61 ± 0.08bA | 0.35 ± 0.13aA | 0.35 ± 0.08aA |
| Alcohols | Ethyl alcohol | 20.43 ± 3.93aA | 21.92 ± 0.86aA | 45.16 ± 3.93cA | 22.53 ± 3.56aA | 35.77 ± 3.93bAB |
| | 2-Butanol | 0.04 ± 0.03aA | 0.05 ± 0.02aA | 1.47 ± 0.03aA | 5.50 ± 1.25aB | 22.08 ± 0.03bB |
| | Isopentyl alcohol | 3.27 ± 0.61aA | 2.87 ± 0.66aA | 8.25 ± 0.61bB | 3.42 ± 0.45aA | 1.96 ± 0.61aA |
| | 1-Hexanol | 2.47 ± 1.79aA | 3.83 ± 2.36abA | 3.09 ± 1.79abA | 6.41 ± 2.53bA | 4.17 ± 1.79abA |
| | 2-Octen-1-ol | 0.08 ± 0.03aA | 0.06 ± 0.01aA | 0.09 ± 0.03aA | 0.09 ± 0.00aA | 0.23 ± 0.03bB |
| Aldehydes | Hexanal | 22.50 ± 8.27aA | 15.92 ± 3.01aA | 31.41 ± 8.27aA | 17.35 ± 10.33aA | 57.40 ± 8.27bB |
| | Nonanal | 0.33 ± 0.11aA | 0.41 ± 0.01aA | 1.13 ± 0.12bA | 0.46 ± 0.31aA | 0.62 ± 0.11aA |
| | Heptanal | 0.53 ± 0.17aA | 0.18 ± 0.16aA | 0.49 ± 0.17aA | 0.17 ± 0.15aA | 0.20 ± 0.17aA |
| | Octanal | 0.24 ± 0.07abA | 0.13 ± 0.10aA | 0.32 ± 0.07bA | 0.20 ± 0.02abA | 0.34 ± 0.07bB |
| | Pentanal | 3.17 ± 1.17aA | 1.96 ± 0.60aA | 3.11 ± 1.17aA | 1.48 ± 1.55aA | 3.16 ± 1.17aA |
| Ketones | Acetoin | 0.01 ± 0.01aA | 0.01 ± 0.01aA | 0.02 ± 0.01aA | 0.05 ± 0.03bA | 0.01 ± 0.01aA |
| | 2-Heptanone | 0.07 ± 0.03abA | 0.03 ± 0.01aA | 0.06 ± 0.03abA | 0.10 ± 0.02bA | 0.08 ± 0.03abA |
| | 2,3-Octanedione | 0.91 ± 0.27aA | 0.62 ± 0.10aA | 1.55 ± 0.27bA | 0.93 ± 0.12aA | 2.85 ± 0.27cB |
| | 2,3-Pentanedione | 0.06 ± 0.06aA | 0.06 ± 0.02aA | 0.25 ± 0.06abA | 0.31 ± 0.16bcA | 0.46 ± 0.06cB |
| | 2-Butanone | 0.14 ± 0.24aA | 0.14 ± 0.13aA | 0.52 ± 0.24aA | 6.10 ± 1.19bB | 5.12 ± 0.24bB |
| Acids | Acetic acid | 8.57 ± 2.56aA | 9.63 ± 4.00aA | 21.45 ± 2.56bA | 5.74 ± 3.45aA | 26.81 ± 2.56bA |
| | Propanoic acid | 0.00 ± 0.00aA | 0.00 ± 0.00aA | 0.02 ± 0.00aB | 0.02 ± 0.01aA | 0.30 ± 0.00bB |
| | Isobutyric acid | 0.00 ± 0.00aA | 0.00 ± 0.00aA | 0.00 ± 0.00aA | 0.06 ± 0.02bA | 0.17 ± 0.00cB |
| | Butanoic acid | 2.15 ± 0.86aA | 2.22 ± 0.98aA | 4.12 ± 0.86bA | 1.04 ± 0.61aA | 4.84 ± 0.86bB |
| | Hexanoic acid | 1.18 ± 0.56abA | 1.19 ± 0.80abA | 1.25 ± 0.56abA | 0.60 ± 0.34aA | 2.58 ± 0.56bB |
| Sulphur compounds | Dimethyl sulfone | 0.05 ± 0.01abA | 0.05 ± 0.02abA | 0.08 ± 0.01bcA | 0.03 ± 0.03aA | 0.09 ± 0.01cA |
| Hydrocarbon | 2-Octene | 1.50 ± 0.60aB | 1.52 ± 1.72aA | 0.01 ± 0.60aA | 0.00 ± 0.00aA | 0.00 ± 0.60aA |
| | | **May** | | | | |
| Class | Volatile compounds (ppb) (µg/kg) | **T4** | **T8** | **T15** | **T30** | **T50** |
| Esters | Ethyl propanoate | 0.11 ± 0.08aB | 0.06 ± 0.02aB | 0.05 ± 0.06aA | 0.05 ± 0.06aA | 0.01 ± 0.00aA |
| | Ethyl isovalerate | 0.33 ± 0.13bcB | 0.25 ± 0.06abB | 0.37 ± 0.08bcB | 0.43 ± 0.12cB | 0.14 ± 0.01aB |
| | Ethyl lactate | 1.18 ± 0.37bA | 1.14 ± 0.41bAB | 1.66 ± 0.20bB | 1.47 ± 0.48bB | 0.46 ± 0.13aA |
| | Methyl hexanoate | 1.18 ± 0.45aA | 1.30 ± 0.16abB | 2.15 ± 0.22cA | 2.00 ± 0.67bcA | 0.73 ± 0.21aA |
| | Butanoic acid, ethyl ester | 0.85 ± 0.17bcAB | 0.64 ± 0.21bAB | 1.00 ± 0.14cA | 0.86 ± 0.20bcB | 0.26 ± 0.06aA |
| | Methyl butyrate | 0.03 ± 0.00aA | 0.03 ± 0.00aA | 0.04 ± 0.01bB | 0.04 ± 0.01bA | 0.02 ± 0.01aA |
| | Methyl propionate | 0.09 ± 0.03aB | 0.08 ± 0.01aA | 0.12 ± 0.02abA | 0.14 ± 0.04bA | 0.08 ± 0.01aA |
| Ethers | 2-Butoxyethanol | 0.43 ± 0.12bB | 0.32 ± 0.09abB | 0.41 ± 0.07bA | 0.48 ± 0.13bA | 0.22 ± 0.05aA |
| Alcohols | Ethyl alcohol | 44.25 ± 11.02bcB | 31.45 ± 3.21abB | 48.15 ± 1.78cA | 45.00 ± 14.60bcB | 17.09 ± 1.41aA |
| | 2-Butanol | 0.08 ± 0.06aA | 0.07 ± 0.02aA | 0.23 ± 0.06abA | 0.50 ± 0.13bA | 0.31 ± 0.28abA |
| | Isopentyl alcohol | 7.28 ± 0.73cC | 4.19 ± 0.36bB | 6.34 ± 1.06cAB | 5.95 ± 1.82bcB | 2.14 ± 0.17aA |
| | 1-Hexanol | 2.82 ± 1.90abA | 4.31 ± 2.00abA | 7.28 ± 3.54bA | 6.26 ± 3.81abA | 1.32 ± 0.69aA |
| | 2-Octen-1-ol | 0.14 ± 0.06bA | 0.11 ± 0.04abA | 0.12 ± 0.03abA | 0.11 ± 0.04abA | 0.05 ± 0.01aA |
| Aldehydes | Hexanal | 36.71 ± 17.52abA | 39.75 ± 7.63abC | 45.78 ± 9.23bA | 52.29 ± 20.78bAB | 17.68 ± 4.17aA |

| | | | | | | |
|---|---|---|---|---|---|---|
| | Nonanal | 1.50 ± 0.51bB | 0.63 ± 0.10aB | 0.63 ± 0.04aA | 0.67 ± 0.30aA | 0.30 ± 0.06aA |
| | Heptanal | 0.90 ± 0.43abA | 0.91 ± 0.26abB | 0.98 ± 0.11bA | 1.02 ± 0.51bAB | 0.33 ± 0.04aA |
| | Octanal | 0.51 ± 0.17bA | 0.38 ± 0.03abB | 0.49 ± 0.07bA | 0.49 ± 0.26bA | 0.14 ± 0.01aA |
| | Pentanal | 3.66 ± 1.77abA | 6.21 ± 1.33bB | 5.74 ± 1.05bA | 6.62 ± 2.91bAB | 1.56 ± 0.55aA |
| Ketones | Acetoin | 0.21 ± 0.04bB | 0.03 ± 0.00aB | 0.02 ± 0.01aA | 0.01 ± 0.01aA | 0.01 ± 0.00aA |
| | 2-Heptanone | 0.10 ± 0.02bA | 0.09 ± 0.01bA | 0.09 ± 0.01bA | 0.10 ± 0.03bA | 0.05 ± 0.00aA |
| | 2,3-Octanedione | 1.78 ± 0.46abA | 1.47 ± 0.32abB | 1.95 ± 0.14bAB | 2.13 ± 0.86bA | 0.95 ± 0.09aA |
| | 2,3-Pentanedione | 0.42 ± 0.08cB | 0.25 ± 0.08abB | 0.30 ± 0.01abcB | 0.34 ± 0.15bcA | 0.16 ± 0.03aA |
| | 2-Butanone | 1.10 ± 0.49bB | 0.61 ± 0.24abB | 0.57 ± 0.17abA | 0.42 ± 0.27aA | 0.40 ± 0.07aA |
| Acids | Acetic acid | 5.19 ± 2.46aA | 13.19 ± 2.57abcA | 22.11 ± 6.75bcA | 24.98 ± 15.23cB | 7.86 ± 1.56abA |
| | Propanoic acid | 0.00 ± 0.00aB | 0.00 ± 0.00aAB | 0.00 ± 0.00aA | 0.01 ± 0.00aA | 0.00 ± 0.00aA |
| | Isobutyric acid | 0.07 ± 0.02aA | 0.14 ± 0.04aB | 0.19 ± 0.04aB | 0.44 ± 0.18bB | 0.16 ± 0.04aB |
| | Butanoic acid | 2.57 ± 1.33abA | 2.90 ± 0.12abA | 5.21 ± 1.21bA | 4.92 ± 2.73bB | 1.54 ± 0.14aA |
| | Hexanoic acid | 2.46 ± 1.95aA | 1.26 ± 0.10aA | 2.34 ± 1.38aA | 2.52 ± 1.23aB | 0.43 ± 0.05aA |
| Sulphur compounds | Dimethyl sulfone | 0.02 ± 0.01aA | 0.02 ± 0.01aA | 0.09 ± 0.03bA | 0.09 ± 0.07bAB | 0.04 ± 0.02abA |
| Hydrocarbon | 2-Octene | 0.00 ± 0.00aA | 0.00 ± 0.00aA | 0.00 ± 0.00aA | 0.00 ± 0.00aA | 0.02 ± 0.04aA |

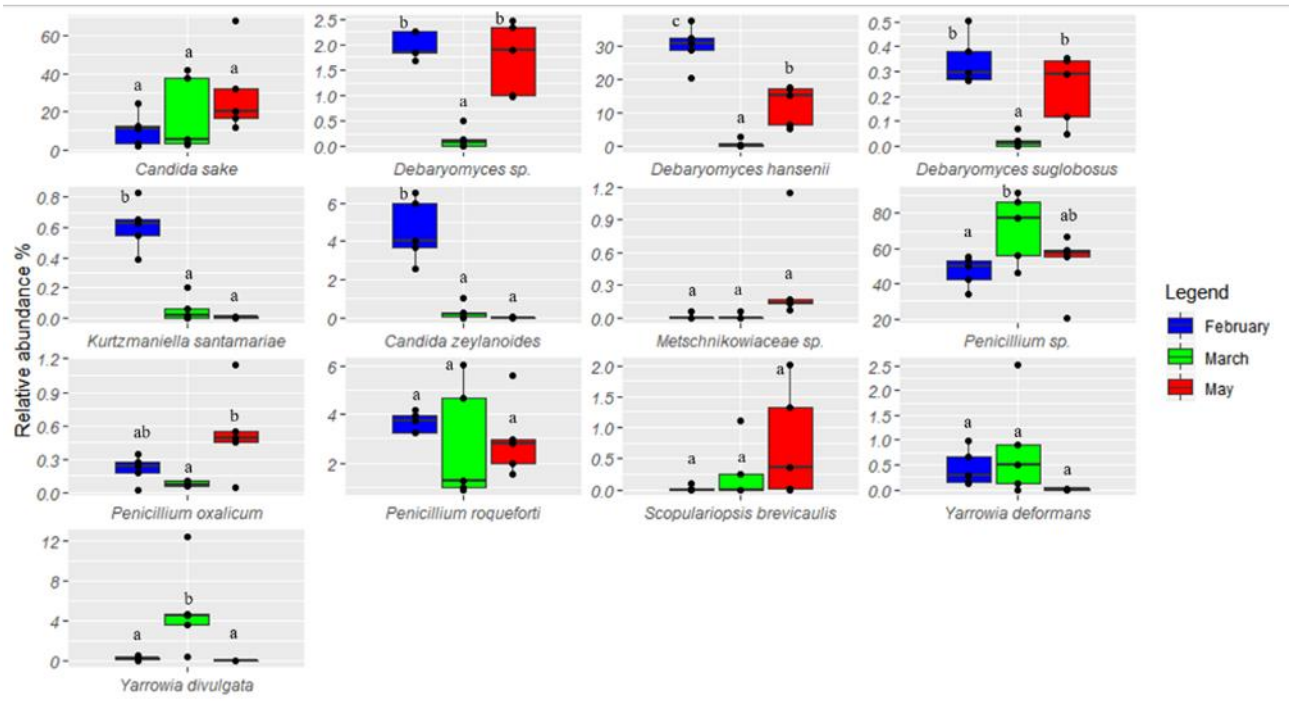
**Supplementary Table 2:** Viable counts of different microbial groups in the three different batches: February, March, May. Different letters in the same column and corresponding to the same time of ripening indicate significant differences ($P < 0.05$) between the three batches.

| | | Mean ± SD | Mean ± SD (log CFU g -1) |
|---|---|---|---|
| **Time (Day)** | **Batch** | **pH** | **LAB** |
| 4 | February | 5.54 ± 0.06b | 8.79 ± 0.10a |
| | March | 4.93 ± 0.03a | 8.99 ± 0.10ab |
| | May | 4.94 ± 0.02a | 9.16 ± 0.14b |
| 8 | February | 5.33 ± 0.01c | 9.31 ± 0.07b |
| | March | 4.95 ± 0.02b | 8.66 ± 0.23a |
| | May | 4.85 ± 0.01a | 9.29 ± 0.13b |
| 15 | February | 5.21 ± 0.54a | 9.73 ± 0.16b |
| | March | 4.99 ± 0.01a | 9.27 ± 0.20a |
| | May | 5.19 ± 0.02a | 9.35 ± 0.02a |
| 30 | February | 5.14 ± 0.03a | 9.2 ± 0.04a |
| | March | 5.39 ± 0.1b | 9.21 ± 0.15a |
| | May | 5.19 ± 0.1a | 10.01 ± 0.80a |
| 50 | February | 5.29 ± 0.03a | 8.97 ± 0.31a |
| | March | 5.72 ± 0.18b | 8.89 ± 0.08a |
| | May | 5.35 ± 0.1a | 9.71 ± 0.09b |

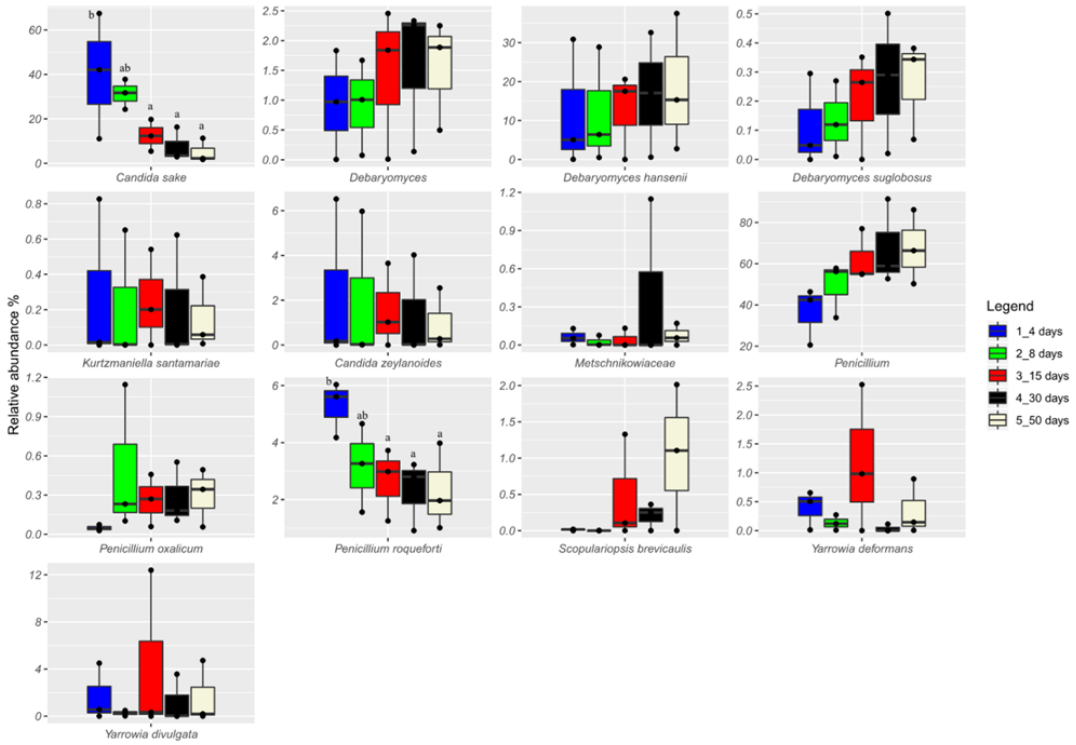
## 8.2. Supplementary Materials Chapter 4



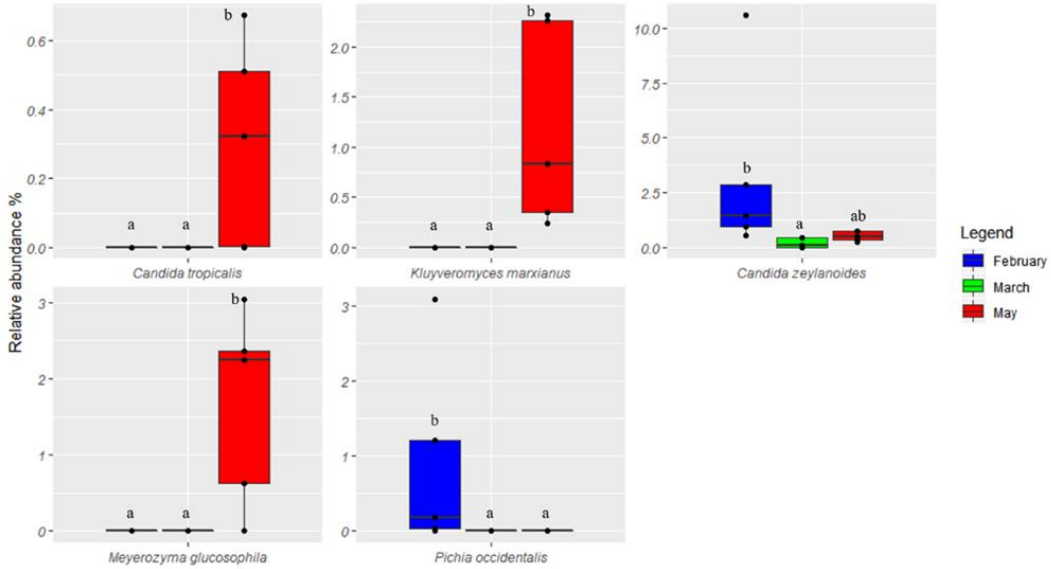

**Supplementary Figure1:** Boxplot showing the alpha diversity index (Chao, Shannon index and observed species) for casing samples between the three batches. Different letters indicate significant differences between index (univariate ANOVA followed by a Duncan post-hoc test : FDR corrected p<0.05).



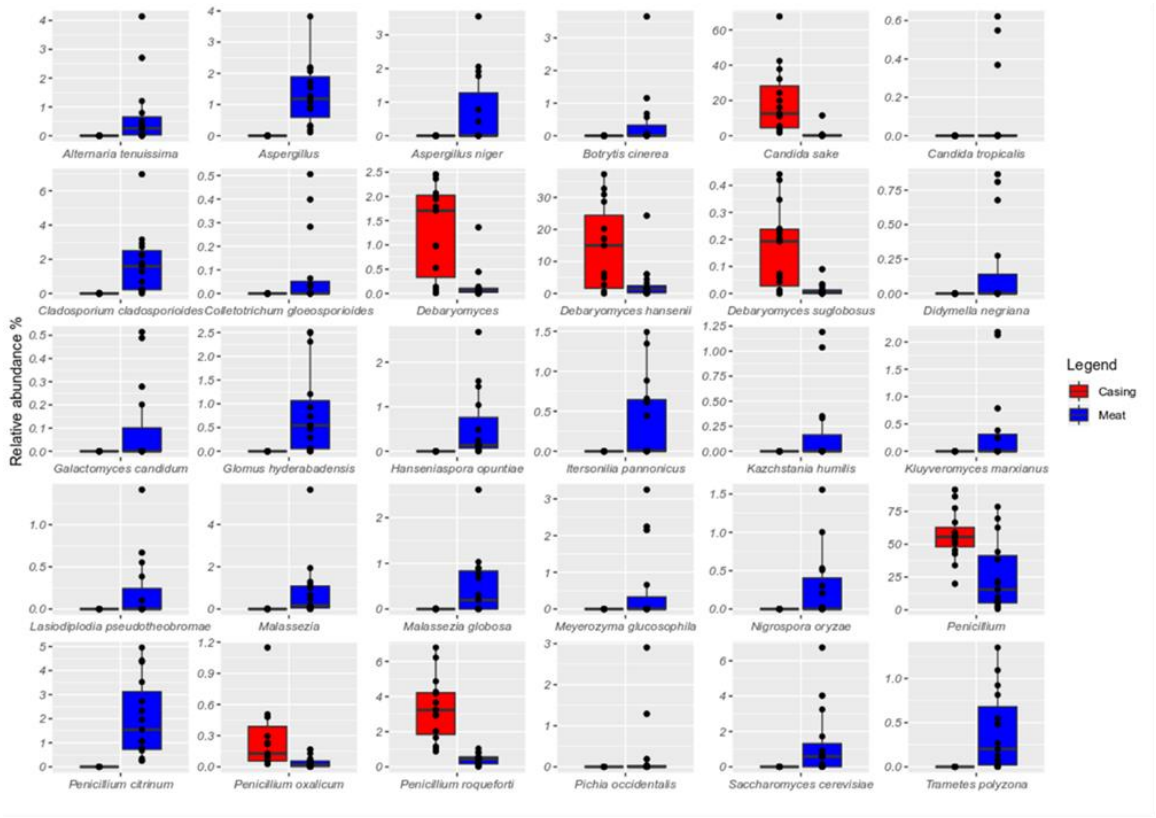

**Supplementary Figure2:** Boxplots showing the relative abundances of the different fungal species present in the casing mycobiota of the three studied batches of Salame Piemonte. Different letters indicate significant differences between groups (univariate ANOVA followed by a Duncan post-hoc test : FDR corrected p<0.05).




**Supplementary Figure3:** Boxplot showing the relative abundances of the different fungal species present in the casing mycobiota of the three studied batches of Salame Piemonte as a function of maturation time. Different letters indicate significant differences between groups (univariate ANOVA followed by a Duncan post-hoc test : FDR corrected $p<0.05$).




**Supplementary Figure4:** Boxplots showing taxa of the meat mycobiota with significantly different relative abundances in the three studied batches of Salame Piemonte. Different letters indicate significant differences between groups (univariate ANOVA followed by a Duncan post-hoc test : FDR-corrected $p<0.05$).




**Supplementary Figure5:** Boxplots showing taxa of the meat and casing mycobiota of Salame Piemonte with significantly different relative abundances (Pairwise comparisons using Wilcoxon rank sum test : FDR as P value adjustment method).

**Supplementary Table1**: Method performance characteristics for metabolite quantification from fermented sausages.

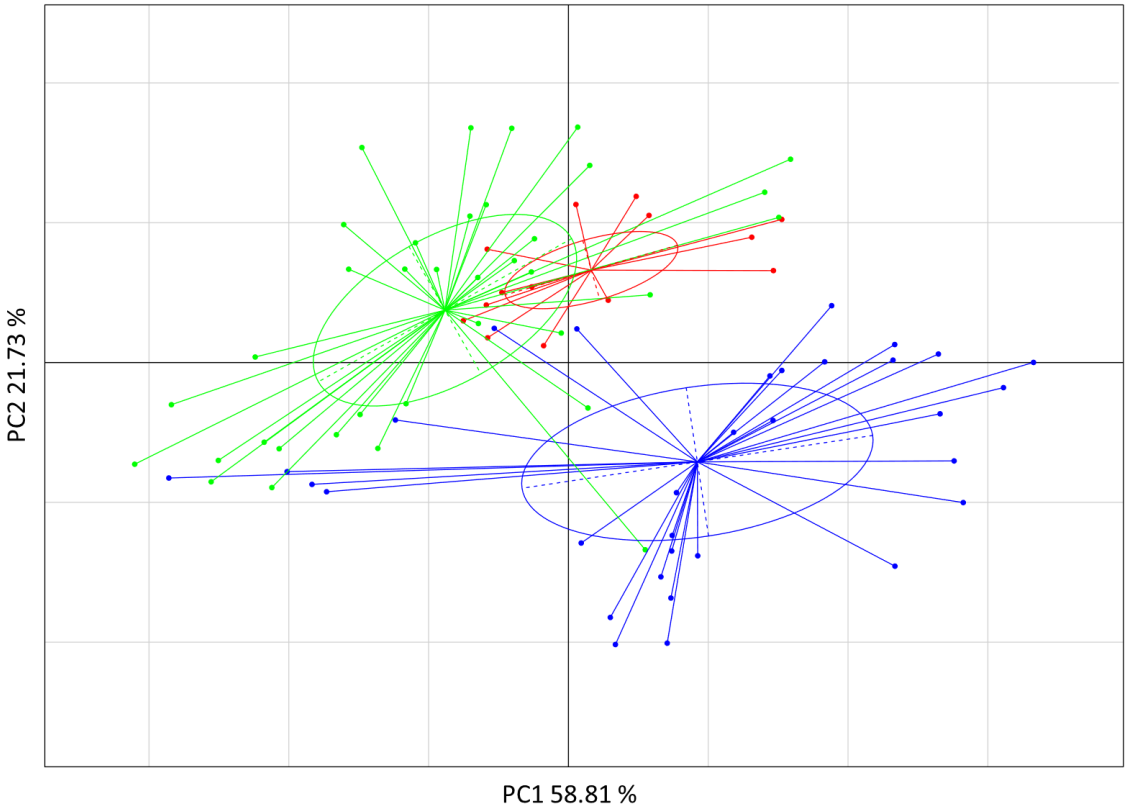
| Compound | Formula | RT (min) | Quantifier Ion (Q1) (m/z) | Qualifier Ion (Q2) (m/z) | $R^2$ | DL ($ng.g^{-1}$) | QL ($ng.g^{-1}$) | ESI |
|---|---|---|---|---|---|---|---|---|
| PAT | $C_7H_6O_4$ | 0.8 | 153.0193 | 177.0193 | 0.999 | 6405.01 | 19409.12 | - |
| (ISO)FUMI A | $C_{18}H_{22}N_2O_2$ | 2.3 | 299.1751 | 322.1573 | 0.996 | 23.31 | 76.70 | + |
| CIT | $C_{13}H_{14}O_5$ | 10.9 | 251.0914 | 273.0733 | 0.998 | 72.35 | 219.26 | + |
| EREM B | $C_{15}H_{20}O_3$ | 11.4 | 249.1484 | 271.1252 | 0.999 | 82.91 | 251.24 | + |
| MELEA | $C_{23}H_{23}N_5O_4$ | 12.5 | 434.1823 | 456.1642 | 0.998 | 56.47 | 171.13 | + |
| AFLB1 | $C_{17}H_{12}O_6$ | 13.2 | 313.0707 | 335.0526 | 0.994 | 37.39 | 113.29 | + |
| ROQ C | $C_{22}H_{23}N_5O_2$ | 15.1 | 390.1928 | N/A | 0.977 | 136.78 | 414.47 | + |
| EREM A | $C_{17}H_{22}O_5$ | 16.1 | 307.1561 | 329.1358 | 0.999 | 124.46 | 377.16 | + |
| GRISEO | $C_{17}H_{17}ClO_6$ | 17.9 | 353.0786 | 375.0606 | 0.991 | 30.35 | 91.96 | + |
| MPA | $C_{17}H_{20}O_6$ | 18.1 | 321.1334 | 303.1231 | 0.999 | 121.47 | 368.10 | + |
| CITREO | $C_{23}H_{30}O_6$ | 19.6 | 403.2115 | 425.1935 | 0.999 | 81.83 | 247.97 | + |
| OTA | $C_{20}H_{18}ClNO_6$ | 21.1 | 404.0895 | 426.0715 | 0.995 | 148.31 | 449.44 | + |
| STERIG | $C_{18}H_{12}O_6$ | 21.9 | 325.0707 | 347.0526 | 0.999 | 61.13 | 185.24 | + |
| CPA | $C_{20}H_{20}N_2O_7$ | 23.3 | 337.1547 | 359.1366 | 0.997 | 112.80 | 341.82 | + |
| AND A | $C_{28}H_{38}O_7$ | 23.5 | 485.2541 | N/A | 0.999 | 102.86 | 311.70 | - |
| PEN A | $C_{37}H_{44}ClNO_6$ | 29.1 | 632.2781 | N/A | 0.993 | 198.89 | 602.71 | - |
| PEN G | $C_{16}H_{18}N_2O_4S$ | 9.6 | 335.1080 | 357.0882 | 0.989 | nd | nd | + |

RT: Retention Time; $R^2$: correlation coefficient; DL: Detection Limit; QL: Quantification Limit; ESI: Electrospray Ionization; NA: not applicable; nd: not determined

**Supplementary Table2**: Fungal counts, pH and water activity (aw) during ripening of three Salame Piemonte batches. Lower case letters in the same column indicate significant differences ($P < 0.05$) between sampling times for the different batches; Capital Letters in the same row indicate significant differences ($P < 0.05$) between sampling times in a same batch.

| Time (Day) | Batch | pH | $a_w$ | Fungal counts ($log_{10}$ CFU/$cm^2$) |
|---|---|---|---|---|
| 0 | February | 6.49 ± 0.10bB | 0.96 ± 0.00aD | n.d. |
| | March | 6.03 ± 0.09aD | 0.96 ± 0.01aC | n.d. |
| | May | 5.99 ± 0.04aD | 0.96 ± 0.00aC | n.d. |
| 4 | February | 5.54 ± 0.06bA | 0.96 ± 0.00bD | 5.35 ± 0.748aA |
| | March | 4.93 ± 0.03aA | 0.96 ± 0.00abC | 6.49 ± 0.378bA |
| | May | 4.94 ± 0.02aA | 0.96 ± 0.00abC | 6.77 ± 0.411bA |
| 8 | February | 5.33 ± 0.01cA | 0.96 ± 0.00bD | 6.22 ± 0.521aAB |
| | March | 4.95 ± 0.02bA | 0.95 ± 0.00aBC | 7.59 ± 0.203bC |
| | May | 4.85 ± 0.01aA | 0.95 ± 0.00aC | 7.39 ± 0.714bA |
| 15 | February | 5.21 ± 0.54aA | 0.95 ± 0.00aC | 6.79 ± 0.336aBC |
| | March | 4.99 ± 0.01aA | 0.95 ± 0.01aBC | 7.54 ± 0.208bBC |
| | May | 5.19 ± 0.02aB | 0.95 ± 0.00aC | 7.59 ± 0.332bA |
| 30 | February | 5.14 ± 0.03aA | 0.94 ± 0.00abB | 7.81 ± 0.704aCD |
| | March | 5.39 ± 0.1bB | 0.94 ± 0.00bB | 7.07 ± 0.151aB |
| | May | 5.19 ± 0.1aB | 0.93 ± 0.01aB | 7.71 ± 0.169aA |
| 50 | February | 5.29 ± 0.03aA | 0.91 ± 0.01aA | 7.96 ± 0.480aD |
| | March | 5.72 ± 0.18bC | 0.92 ± 0.01aA | 7.76 ± 0.314aC |
| | May | 5.35 ± 0.1aC | 0.91 ± 0.01aA | 7.58 ± 0.547aA |

## 8.3. Supplementary Materials Chapter 5




**Supplementary Figure 1:** PCA based on acidification rate for LAB REP biotype (February in blue, March in green, May in red). The first component (horizontal) accounts for 58.81% of the variance and the second component (vertical) accounts for 21.73%.

**Supplementary Table 1:** Chemical values (pH and *aw*) and viable counts of different microbial groups in the three different batches: February, March, May. Different letters in the same column and corresponding to the same time of ripening indicate significant differences ($P < 0.05$) between the three batches.

| | | Mean ± SD | | Mean ± SD ($log_{10}$ CFU / g) | | |
|---|---|---|---|---|---|---|
| **Time (Day)** | **Batch** | **pH** | ***aw*** | **LAB** | ***Staphylococcaceae*** | ***Enterobacteriaceae*** |
| 0 | February | 6.49 ± 0.10b | 0.96 ± 0.00a | 3.46 ± 0.14a | 3.53 ± 0.12a | 2.24 ± 0.32ab |
| | March | 6.03 ± 0.09a | 0.96 ± 0.01a | 3.87 ± 0.23ab | 3.44 ± 0.23a | 1.55 ± 0.43a |
| | May | 5.99 ± 0.04a | 0.96 ± 0.00a | 4.01 ± 0.31b | 3.57 ± 0.66a | 2.88 ± 0.61b |
| 4 | February | 5.54 ± 0.06b | 0.96 ± 0.00a | 8.79 ± 0.10a | 5.03 ± 0.35b | 2.16 ± 0.19b |
| | March | 4.93 ± 0.03a | 0.96 ± 0.00a | 8.99 ± 0.10ab | 3.14 ± 0.46a | 1.10 ± 0.17a |
| | May | 4.94 ± 0.02a | 0.96 ± 0.00a | 9.16 ± 0.14b | 3.59 ± 0.11a | 1.20 ± 0.17a |
| 8 | February | 5.33 ± 0.01c | 0.96 ± 0.00b | 9.31 ± 0.07b | 4.14 ± 0.20a | 1.93 ± 0.35a |
| | March | 4.95 ± 0.02b | 0.95 ± 0.00a | 8.66 ± 0.23a | 3.96 ± 0.60a | 1.10 ± 0.17a |
| | May | 4.85 ± 0.01a | 0.95 ± 0.00a | 9.29 ± 0.13b | 3.58 ± 0.47a | 1.16 ± 0.28a |
| 15 | February | 5.21 ± 0.54a | 0.95 ± 0.00a | 9.73 ± 0.16b | 3.82 ± 1.04a | 1.43 ± 0.38ab |
| | March | 4.99 ± 0.01a | 0.95 ± 0.01a | 9.27 ± 0.20a | 3.25 ± 0.38a | 1.50 ± 0.17b |
| | May | 5.19 ± 0.02a | 0.95 ± 0.00a | 9.35 ± 0.02a | 4.38 ± 1.06a | < 1a |
| 30 | February | 5.14 ± 0.03a | 0.94 ± 0.00a | 9.20 ± 0.04a | 3.75 ± 0.27a | 1.39 ± 0.36b |
| | March | 5.39 ± 0.1b | 0.94 ± 0.00a | 9.21 ± 0.15a | 5.49 ± 0.19b | < 1a |
| | May | 5.19 ± 0.1a | 0.93 ± 0.01a | 10.01 ± 0.80a | 3.94 ± 0.40a | < 1a |
| 50 | February | 5.29 ± 0.03a | 0.91 ± 0.01a | 8.97 ± 0.31a | 4.23 ± 0.11b | 1.59 ± 0.36b |
| | March | 5.72 ± 0.18b | 0.92 ± 0.01a | 8.89 ± 0.08a | 3.09 ± 0.21a | < 1a |
| | May | 5.35 ± 0.1a | 0.91 ± 0.01a | 9.71 ± 0.09b | 3.09 ±0.06a | < 1a |

**Supplementary Table 2:** Summary of physiological values of LAB isolates: (A) different letters in the same line indicate significant differences ($P < 0.05$) between the three batches; (B) different letters in the same line indicate significant differences ($P < 0.05$) between the most isolated three LAB species.

| **A** | Means ± SD | | |
|---|---|---|---|
| **Conditions:** | **February** | **March** | **May** |
| Growth rate at 30°C | 0.43 ± 0.14a | 0.33 ± 0.27a | 0.42 ± 0.07a |
| Growth rate at 20°C | 0.18 ± 0.09a | 0.15 ± 0.10a | 0.25 ± 0.03b |
| Growth rate at 15°C | 0.07 ± 0.05b | 0.03 ± 0.05a | 0.04 ± 0.04ab |
| Growth rate at 10°C | 0.02 ± 0.02a | 0.07 ± 0.18a | 0.01 ± 0.01a |
| pH value at 30°C | 5.44 ± 0.38a | 5.82 ± 0.28b | 5.34 ± 0.28a |
| pH value at 20°C | 5.10 ± 0.09a | 6.15 ± 0.06c | 6.10 ± 0.04b |
| pH value at 15°C | 5.91 ± 0.17b | 5.83 ± 0.20ab | 5.77 ± 0.08a |
| pH value at 10°C | 6.11 ± 0.07a | 6.20 ± 0.10b | 6.21 ± 0.02b |
| Growth rate with 2% NaCl | 0.15 ± 0.25a | 0.54 ± 0.17b | 0.54 ± 0.04b |
| Growth rate with 3% NaCl | 0.15 ± 0.27a | 0.53 ± 0.18b | 0.57 ± 0.12b |
| Growth rate with 4% NaCl | 0.27 ± 0.27a | 0.34 ± 0.21a | 0.36 ± 0.16a |
| | | | |
| **B** | ***L. sakei*** | ***P. pentosaceus*** | ***L. curvatus*** |
| Growth rate at 30°C | 0.38 ± 0.18b | 0.45 ± 0.11b | 0.29 ± 0.27a |
| Growth rate at 20°C | 0.2 ± 0.10b | 0.20 ± 0.05b | 0.14 ± 0.10a |

| | | | |
|---|---|---|---|
| Growth rate at 15°C | 0.03 ± 0.05a | 0.10 ± 0.02b | 0.05 ± 0.05a |
| Growth rate at 10°C | 0.03 ± 0.12a | 0.03 ± 0.01a | 0.08 ± 0.21a |
| pH value at 30°C | 5.65 ± 0.29b | 4.96 ± 0.15a | 5.84 ± 0.25c |
| pH value at 20°C | 6.09 ± 0.08b | 5.96 ± 0.06a | 6.15 ± 0.07c |
| pH value at 15°C | 5.89 ± 0.17b | 5.75 ± 0.09a | 5.83 ± 0.21b |
| pH value at 10°C | 6.17 ± 0.09b | 6.12 ± 0.08a | 6.21 ± 0.08b |
| Growth rate with 2% NaCl | 0.37 ± 0.26b | 0.25 ± 0.30a | 0.59 ± 0.12c |
| Growth rate with 3% NaCl | 0.41 ± 0.28b | 0.23 ± 0.30a | 0.52 ± 0.21b |
| Growth rate with 4% NaCl | 0.24 ± 0.22a | 0.54 ± 0.06c | 0.39 ± 0.18b |

**Supplementary Table 3:** Antibiotic susceptibility testing for 6 LAB and 1 CNS strains with 12 antibiotics.

| **MIC (ug/ml)** | | | | | | | |
|---|---|---|---|---|---|---|---|
| Values in tables correspond to minimal inhibitory concentrations (mg / L) and, in parentheses, microbiological cut-off values (mg / L) according to EFSA (2012). Abbreviations: nr, not required; nd, not determined. | | | | | | | |
| **Species** | **Sample Code** | **Kanamycin** | **Streptomycin** | **Tetracyclin** | **Erythromycin** | **Clindamycin** | **Chloramphenicol** |
| *Pediococcus pentosaceus* | S8QM | 256 (64) | 128 (64) | 8 (8) | 0.5 (1) | 0.25 (1) | 2.6 (4) |
| | S4XNM | 16 (64) | 53.3 (64) | 0.4 (8) | 0.3 (1) | 0.2 (1) | 1.7 (4) |
| *Latilactobacillus sakei* | S29BM | 32 (64) | 64 (64) | 0.7 (8) | 0.4 (1) | 0.2 (1) | 2 (4) |
| | S30ZEM | 26.7 (64) | 53.3 (64) | 0.5 (8) | 0.4 (1) | 0.1 (1) | 2 (4) |
| *Staphylococcus xylosus* | S30XIS | 2 (16) | 0.5 (8) | 0.25 (2) | 0.3 (0.5) | 0.5 (0.25) | 8 (2) |
| | S8HS | 2 (16) | 0.5 (8) | 0.25 (2) | 0.3 (0.5) | 0.5 (0.25) | 4 (2) |
| *Lactiplantibacillus plantarum* | LMG6907 | 128 (64) | 64 (64) | 8 (4) | 0.8 (1) | 3.3 (1) | 4 (4) |
| **MIC (ug/ml)** | | | | | | | |
| Values in tables correspond to minimal inhibitory concentrations (mg / L) and, in parentheses, microbiological cut-off values (mg / L) according to EFSA (2012). Abbreviations: nr, not required; nd, not determined. | | | | | | | |
| **Species** | **Sample Code** | **Ampicilline** | **Neomycin** | **Vancomycine** | **Trimethoprim** | **Oxytetracycline** | **Rifampicin** |
| *Pediococcus pentosaceus* | S8QM | 2.6 (4) | 32 (nd) | >128 (nr) | 10.6 (nd) | 21.3 (nd) | 0.43 (nd) |
| | S4XNM | 1.2 (4) | 16 (nd) | >128 (nr) | 6.7 (nd) | 1.3 (nd) | 0.125 (nd) |
| *Latilactobacillus sakei* | S29BM | 2 (4) | 16 (nd) | >128 (nr) | 10.7 (nd) | 2 (nd) | 0.125 (nd) |
| | S30ZEM | 1.3 (4) | 16 (nd) | >128 (nr) | 6.7 (nd) | 1.7 (nd) | 0.125 (nd) |
| *Staphylococcus xylosus* | S30XIS | 0.5 (1) | 1 (nd) | 1.5 (2) | 0.8 (nd) | 0.7 (nd) | 0.125 (nd) |
| | S8HS | 0.6 (1) | 0.5 (nd) | 0.25 (2) | 0.7 (nd) | 0.5 (nd) | 0.125 (nd) |
| *Lactiplantibacillus plantarum* | LMG6907 | 0.1 (4) | 10.7 (nd) | >128 (nr) | 2.3 (nd) | 21.3 (nd) | 1 (nd) |

**Supplementary Table 4:** Chemical values (pH) and viable counts of different microbial groups (CNS and LAB) in the seven autochthonous starter culture (ASC) and the control one. Different letters in the same column and corresponding to the same time of ripening indicate significant differences ($P < 0.05$) between the ASCs.

| | | Mean ± SD ($log_{10}$ CFU / g) | | Mean ± SD |
|---|---|---|---|---|
| **Time (Day)** | | **CNS** | **LAB** | **pH** |
| **0** | ASC 1 | 8.28 ± 0.19d | 8.23 ± 0.04e | 5.88 ± 0.00b |
| | ASC 2 | 8.13 ± 0.03cd | 7.77 ± 0.03d | 6.05 ± 0.00d |
| | ASC 3 | 8.46 ± 0.06d | 8.29 ± 0.02e | 5.76 ± 0.00a |
| | ASC 4 | 7.55 ± 0.08b | 7.41 ± 0.08b | 5.92 ± 0.1bc |
| | ASC 5 | 7.76 ± 0.08bc | 7.66 ± 0.04cd | 6.02 ± 0.03cd |
| | ASC 6 | 7.69 ± 0.14b | 7.63 ± 0.05cd | 6.02 ± 0.02cd |
| | ASC 7 | 7.52 ± 0.16b | 7.46 ± 0.15bc | 5.95 ± 0.04bcd |
| | Control | 5.91 ± 0.4a | 6.3 ± 0.19a | 5.93 ± 0.06bc |
| **2** | ASC 1 | 8.23 ± 0.07c | 8.88 ± 0.14b | 5.13 ± 0.00a |
| | ASC 2 | 8.29 ± 0.08cd | 8.95 ± 0.08b | 5.09 ± 0.00a |
| | ASC 3 | 8.58 ± 0.01d | 9.44 ± 0.12c | 5.07 ± 0.00a |
| | ASC 4 | 7.63 ± 0.22b | 7.83 ± 0.1a | 5.98 ± 0.03c |
| | ASC 5 | 7.67 ± 0.2b | 7.68 ± 0.19a | 6.41 ± 0.46d |
| | ASC 6 | 7.58 ± 0.23b | 7.56 ± 0.25a | 6.13 ± 0.08cd |
| | ASC 7 | 7.44 ± 0.14b | 7.62 ± 0.21a | 6.09 ± 0.12cd |
| | Control | 6.51 ± 0.27a | 8.8 ± 0.19b | 5.52 ± 0.05b |
| **12** | ASC 1 | 8.67 ± 0.47c | 9.29 ± 0.2b | 5.22 ± 0.00a |
| | ASC 2 | 8.52 ± 0.35c | 9.17 ± 0.08b | 5.18 ± 0.00a |
| | ASC 3 | 8.4 ± 0.08c | 9.35 ± 0.29b | 5.16 ± 0.00a |
| | ASC 4 | 7.16 ± 0.49b | 9.17 ± 0.13b | 5.18 ± 0.05a |
| | ASC 5 | 7.26 ± 0.44b | 9.17 ± 0.09b | 5.42 ± 0.17b |
| | ASC 6 | 7.34 ± 0.19b | 9.22 ± 0.08b | 5.46 ± 0.05b |
| | ASC 7 | 7.24 ± 0.41b | 8.86 ± 0.11a | 5.25 ± 0.03a |
| | Control | 6.42 ± 0.00a | 9.67 ± 0.12c | 5.14 ± 0.03a |
| **15** | ASC 1 | 8.7 ± 0.51c | 9.35 ± 0.18cd | 5.25 ± 0.00a |
| | ASC 2 | 8.93 ± 0.29c | 9.02 ± 0.19ab | 5.21 ± 0.00a |
| | ASC 3 | 8.77 ± 0.62c | 9.24 ± 0.2bcd | 5.22 ± 0.00a |
| | ASC 4 | 6.74 ± 0.24b | 9.28 ± 0.14cd | 5.27 ± 0.04a |
| | ASC 5 | 6.75 ± 0.39b | 9.09 ± 0.04abc | 5.24 ± 0.05a |
| | ASC 6 | 6.89 ± 0.11b | 9.25 ± 0.08bcd | 5.24 ± 0.09a |
| | ASC 7 | 7.09 ± 0.32b | 8.91 ± 0.07a | 5.22 ± 0.05a |
| | Control | 5.83 ± 0.38a | 9.48 ± 0.09d | 5.21 ± 0.00a |

**Supplementary Table 5:** Volatile compounds (ppb) determined on sausages of the seven different autochthonous starter culture (ASC) and the control one. Different letters in the same line indicate significant differences ($P < 0.05$) between the different ASCs.

| | Volatile compounds concentrations (ppb) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **ASC 1** | **ASC 2** | **ASC 3** | **ASC 4** | **ASC 5** | **ASC 6** | **ASC 7** | **Control** |
| **Amino acid metabolism** | | | | | | | | |
| 2-methyl-Butanal | 3.26 ± 1.44b | 1.99 ± 1.45ab | 1.64 ± 1.39ab | 0.59 ± 0.31a | 2.33 ± 3.41ab | 0.37 ± 0.11a | 1.35 ± 1.58ab | 0.12 ± 0.21a |
| 3-methyl-Butanoic acid | 53.91 ± 16.78a | 46.32 ± 46.97a | 24.28 ± 27.39a | 22.96 ± 17.84a | 35.73 ± 27.3a | 26.5 ± 13.7a | 42.01 ± 41.09a | 18.24 ± 3.93a |
| Dimethyl sulfone | 6.74 ± 3.70b | 3.85 ± 2.23a | 3.59 ± 1.3a | 1.73 ± 0.69a | 2.75 ± 1.42a | 1.73 ± 0.43a | 2.52 ± 1.28a | 2.09 ± 1.03a |
| Butanoic acid, methyl ester | 6.26 ± 6.12a | 16.24 ± 11.74ab | 12.45 ± 9.13ab | 17.26 ± 5.45ab | 30.5 ± 13.87c | 14.81 ± 2.1ab | 21.74 ± 10.68bc | 16.29 ± 5.55ab |
| Methyl isobutyrate | 8.53 ± 6.25a | 5.65 ± 3.15a | 4.18 ± 3.47a | 9.73 ± 5.57a | 13.67 ± 11.75a | 11.49 ± 7.14a | 12.4 ± 8.78a | 3.94 ± 0.14a |
| 3-methyl-Butanal | 8.3 ± 3.29b | 5.64 ± 4.35ab | 4.04 ± 4.06ab | 2.36 ± 0.75ab | 7.82 ± 7.96ab | 1.99 ± 1.62a | 5.2 ± 5.3ab | 1.98 ± 1.29a |
| Diacetyl | 61.38 ± 15.57c | 36.48 ± 22.22abc | 43.93 ± 20.4abc | 24.07 ± 12.84ab | 26.05 ± 16.46ab | 18.71 ± 5.4a | 44.03 ± 25.29abc | 46.64 ± 13.13bc |
| 1-Butanol | 3.86 ± 6.98b | 1.11 ± 1.24ab | 1.46 ± 1.75ab | 0.00 ± 0.00a | 0.28 ± 0.7a | 0.00 ± 0.00a | 0.00 ± 0.00a | 0.00 ± 0.00a |
| 2-methyl-1-Propanol | 20.41 ± 18.8b | 8.86 ± 6.32a | 10.94 ± 8.44a | 3.32 ± 1.11a | 4.19 ± 1.71a | 3.18 ± 1.04a | 4.76 ± 2.41a | 3.16 ± 0.81a |
| Acetoin | 980.89 ± 322.83c | 636.56 ± 362.57bc | 764.27 ± 348.85c | 232.23 ± 162.15a | 203.05 ± 172.86a | 142.53 ± 53.55a | 393.03 ± 303.58ab | 656.69 ± 217.74bc |
| Isobutyric acid | 23.29 ± 9.73a | 22.61 ± 22.48a | 11.58 ± 10.54a | 21.1 ± 16.54a | 25.29 ± 22a | 24.22 ± 13.94a | 30.44 ± 26.37a | 11.29 ± 2.96a |
| Phenylethyl Alcohol | 12.94 ± 4.46b | 9.18 ± 6.63ab | 7.38 ± 3.45ab | 5.84 ± 2.8a | 11.15 ± 6.63ab | 5.85 ± 1.68a | 10.02 ± 5.43ab | 8.06 ± 3.11ab |
| **Carbohydrate metabolism** | | | | | | | | |
| Ethyl alcohol | 1137.61 ± 478.98d | 875.54 ± 629.86bcd | 954.89 ± 403.19cd | 312.02 ± 89.6a | 811.29 ± 217.34abcd | 400.42 ± 305.08ab | 441.75 ± 264.81ab | 493.84 ± 443.86abc |
| 2-Butanone | 573.69 ± 220.87d | 201.93 ± 166.26c | 179.21 ± 90.53bc | 56.1 ± 38.46ab | 57.92 ± 37.96ab | 26.52 ± 6.73a | 40.5 ± 21.6a | 63.03 ± 13.71ab |
| Acetic acid | 814.11 ± 469.51c | 579.76 ± 378.19bc | 518.5 ± 152.02b | 111.38 ± 89.73a | 218.96 ± 180.15a | 105.37 ± 48.35a | 127.95 ± 82.38a | 143.35 ± 73.72a |
| Methyl acetate | 120.18 ± 73.09abc | 189.4 ± 118.88c | 141.68 ± 129.81bc | 31.95 ± 27.61ab | 114.76 ± 120.65abc | 25.65 ± 16.45ab | 52.54 ± 44.68ab | 18.92 ± 13.74a |
| Isopentyl alcohol | 341.78 ± 198.23c | 210 ± 138.84b | 222.92 ± 101.06b | 55.92 ± 19.12a | 74.44 ± 27.77a | 54.2 ± 18.72a | 83.76 ± 43.05a | 50.87 ± 13.46a |
| Butanoic acid | 27.67 ± 17.86a | 22.42 ± 18.29a | 18.17 ± 5.71a | 20.66 ± 13.57a | 33.33 ± 13.51a | 15.82 ± 7.69a | 23.24 ± 14.42a | 20.19 ± 9.05a |
| Ethyl lactate | 25.49 ± 15.67ab | 16.35 ± 11.32ab | 18.28 ± 8.84ab | 9.89 ± 4.99a | 29.88 ± 15.88b | 14.61 ± 17.06ab | 16.98 ± 14.77ab | 16.63 ± 15.06ab |
| 1-hydroxy-2-Propanone | 17.12 ± 4.83a | 14.46 ± 9.45a | 16.34 ± 7.11a | 17.26 ± 8.75a | 11.01 ± 8.62a | 15.18 ± 4.49a | 35 ± 17.71b | 17.07 ± 5.25a |
| **Esterase activity** | | | | | | | | |
| Ethyl Acetate | 169.7 ± 101.47b | 132.54 ± 94.44b | 151.76 ± 71.81b | 37.64 ± 13.28a | 146.9 ± 82.87b | 46.15 ± 35.28a | 41.54 ± 25.62a | 35.02 ± 14.25a |
| Butanoic acid, ethyl ester | 0.00 ± 0.00a | 0.00 ± 0.00a | 0.00 ± 0.00a | 4.48 ± 2.56ab | 15.58 ± 5.95c | 5.55 ± 5.22b | 6.32 ± 4.64b | 6.04 ± 5.03b |
| Ethyl 2-methylbutanoate | 0.00 ± 0.00a | 0.00 ± 0.00a | 0.00 ± 0.00a | 1.24 ± 0.59b | 3.33 ± 1.38c | 1.61 ± 0.98b | 1.62 ± 1.11b | 0.79 ± 0.6ab |
| Ethyl isovalerate | 0.00 ± 0.00a | 2.23 ± 3.95ab | 1.09 ± 2.89ab | 2.95 ± 1.54ab | 9.19 ± 3.73c | 4.22 ± 2.4b | 4.27 ± 3.03b | 2.43 ± 1.14ab |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Ethyl caproate | 1.88 ± 2.34a | 1.85 ± 1.43a | 1.32 ± 1.12a | 1.32 ± 0.6a | 4.95 ± 2.17b | 1.39 ± 1.38a | 1.89 ± 1.43a | 1.72 ± 1.19a |
| 1-Octen-3-ol | 49.18 ± 40.13b | 13.29 ± 14.96ab | 30.93 ± 18.07ab | 15.69 ± 17.5ab | 0.00 ± 0.00a | 28.62 ± 21.17ab | 51.57 ± 51.95b | 21.27 ± 9.07ab |
| **Lipid metabolism** | | | | | | | | |
| Hexanal | 88.13 ± 95.54b | 47.41 ± 72.57ab | 53.76 ± 50.88ab | 6.49 ± 2.49a | 8.36 ± 5.21a | 5.23 ± 2.13a | 4.65 ± 2.73a | 4.27 ± 1.14a |
| 2-Pentanone | 70.34 ± 23.59d | 36.84 ± 24.12bc | 43.75 ± 28.22c | 15 ± 7.14ab | 14.83 ± 8.15ab | 11.49 ± 3.67a | 24.25 ± 12.1abc | 23.15 ± 5.79abc |
| Methyl caproate | 11.58 ± 10.98a | 18.27 ± 4.74ab | 10.26 ± 13.63a | 14.47 ± 5.4a | 27.84 ± 11.94b | 12.53 ± 1.56a | 16.98 ± 7.69ab | 14.01 ± 5.02a |
| 1-Pentanol | 9.06 ± 6.78ab | 16.98 ± 18.32b | 14.52 ± 11.73ab | 5.42 ± 1.87ab | 7.44 ± 2.99ab | 3.93 ± 2.43a | 7.85 ± 4.68ab | 4.03 ± 0.95a |
| 1-Hexanol | 75.58 ± 58.34b | 80.9 ± 86.76b | 64.69 ± 58.27b | 4.55 ± 1.8a | 7.06 ± 3.46a | 4.2 ± 1.38a | 8.82 ± 4.73a | 5.21 ± 1.76a |
| Nonanal | 4.85 ± 2.78ab | 3.12 ± 2.95a | 2.63 ± 1.11a | 3.15 ± 0.7a | 5.89 ± 2.34b | 3.15 ± 0.96a | 3.4 ± 1.34a | 3.6 ± 2.18ab |
| Hexanoic acid | 0.05 ± 0.11a | 0.00 ± 0.00a | 0.00 ± 0.00a | 13.77 ± 3.48b | 10.18 ± 8.42b | 11.19 ± 6.88b | 8.02 ± 8.57b | 10.62 ± 2.95b |
| Octanoic acid | 0.00 ± 0.00a | 0.00 ± 0.00a | 0.00 ± 0.00a | 7.59 ± 4.89b | 9.07 ± 7.67b | 10.12 ± 8.99b | 8.82 ± 6.2b | 0.36 ± 0.38a |
| Methyl octanoate | 1.79 ± 1.42a | 3.42 ± 1.74a | 2.61 ± 2.96a | 4.82 ± 3.3a | 10.94 ± 8.18b | 3.91 ± 1.65a | 7.01 ± 4.26ab | 3.81 ± 2.36a |
| **Spices** | | | | | | | | |
| Allyl methyl sulfide | 136.84 ± 52.26ab | 60.2 ± 43.77a | 89.34 ± 63.75ab | 123.22 ± 43.2ab | 162.1 ± 70.53b | 117.26 ± 42.74ab | 160.23 ± 86.93b | 75.71 ± 19.5a |
| Styrene | 4.65 ± 2.66b | 2.29 ± 1.56a | 2.57 ± 1.53a | 1.17 ± 0.31a | 1.19 ± 0.57a | 0.99 ± 0.32a | 1.43 ± 0.69a | 1.12 ± 0.56a |
| **Unknown origin** | | | | | | | | |
| Ethyl decanoate | 0.77 ± 0.23ab | 0.46 ± 0.38a | 0.48 ± 0.15a | 1.23 ± 0.51ab | 2.57 ± 1.17c | 1.18 ± 0.5ab | 1.39 ± 0.69b | 1.27 ± 0.48ab |
| Butyrolactone | 12.07 ± 9.5b | 10.65 ± 6.54b | 11.27 ± 5.54b | 1.78 ± 0.77a | 2.61 ± 0.58a | 2.16 ± 0.8a | 3.48 ± 1.78a | 1.98 ± 1.24a |
| 1-Octanol | 0.69 ± 0.60b | 0.69 ± 0.74b | 0.41 ± 0.43ab | 0.09 ± 0.21a | 0.00 ± 0.00a | 0.00 ± 0.00a | 0.00 ± 0.00a | 0.00 ± 0.00a |
| 2-Ethyl-1-hexanol | 11.46 ± 8.10b | 5.85 ± 4.75a | 5.96 ± 4.09a | 2.6 ± 1.23a | 2.68 ± 1.78a | 2.19 ± 0.55a | 3.95 ± 1.87a | 3.34 ± 1.42a |
| Methyl(1-methylethenyl)-Benzene | 6.94 ± 2.70c | 5.25 ± 3.81c | 4.83 ± 2.63bc | 1.87 ± 0.88a | 2.41 ± 1.12ab | 2.09 ± 0.66ab | 2.59 ± 1.17ab | 2.24 ± 0.95ab |
| 2-butoxy-Ethanol | 10.54 ± 8.88a | 6.15 ± 4.32a | 5.24 ± 1.85a | 9.02 ± 4.34a | 8.66 ± 4.9a | 7.11 ± 3.03a | 7.97 ± 4a | 9.69 ± 5.48a |
| Lactic acid, methyl ester | 42.70 ± 9.36bcd | 47.25 ± 12.32d | 46.1 ± 25.12cd | 23.56 ± 11.19abc | 45.02 ± 20.39cd | 21.41 ± 8.41ab | 31.88 ± 20.47abcd | 19.7 ± 5.26a |
| 2-methyl-3-Octanone | 29.3 ± 26.83b | 22.14 ± 26.02b | 20.66 ± 19.82b | 0.00 ± 0.00a | 0.00 ± 0.00a | 0.7 ± 1.71a | 0.00 ± 0.00a | 0.00 ± 0.00a |
| 6-Methyl-5-heptene-2-one | 2.02 ± 1.76b | 0.97 ± 0.77a | 0.87 ± 0.45a | 0.44 ± 0.22a | 0.47 ± 0.19a | 0.44 ± 0.14a | 0.61 ± 0.28a | 0.59 ± 0.26a |

# Chapter 9 - Acknowledgements

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**Titolo:** Selezione di colture starter autoctone per la produzione di Salame Piemonte PGI attraverso l'analisi del microbiota.

**Keywords:** Salsicce fermentate; Selezione colture starter; Microbiota; Mycobiota; Metodi cultura-dipendente; Analisi metagenomiche; Volatiloma

Le proprietà sensoriali degli alimenti fermentati artigianali si basano sulle complesse attività di consorzi microbici. Questo progetto di ricerca mirava ad indagare la diversità, le dinamiche e il ruolo funzionale dei microorganismi coinvolti in uno specifico alimento fermentato mediterraneo, il Salame Piemonte IGP. Un ulteriore obiettivo era quello di sviluppare nuove colture starter composte da ceppi autoctoni per migliorare la qualità complessiva del prodotto. Su tre diversi lotti di salsicce a fermentazione naturale sono stati applicati metodi coltura dipendenti ed indipendenti, che hanno permesso di decifrare la diversità e le dinamiche batteriche e fungine del Salame Piemonte. Questo prodotto è infatti caratterizzato dalla dominanza di un numero limitato di batteri lattici (LAB), stafilococchi coagulasi negativi (CNS) e specie fungine. I salami dei diversi lotti hanno mostrato una diversa composizione volatile ed una forte associazione tra gli attributi che ne definiscono la qualità e i ceppi LAB presenti. Infine, sono state sviluppate e valutate colture starter autoctone per la preparazione di salame su scala pilota che hanno portato a risultati promettenti dimostrando l'interesse dell'uso di tali colture starter autoctone per migliorare le proprietà sensoriali del prodotto.

**Title:** Autochthonous starter culture selection for PGI Salame Piemonte production through the microbiota analysis.

**Keywords:** Fermented sausages; Starter cultures selection; Microbiota; Mycobiota; Culture-dependent methods; Metagenomic approach; Volatilome

The manufacture and sensorial properties of artisanal fermented foods relies on complex and poorly defined microbial consortia activities. This PhD research project aimed at investigating the diversity, dynamics and functional role of the microbiota involved in a specific Mediterranean fermented foods, PGI *Salame Piemonte*. Another aim was to develop new starter cultures composed of autochthonous strains to improve overall product quality. Culture-dependent and -independent methods were applied on three different batches of naturally fermented sausages allowing to decipher bacterial and fungal diversity and dynamics of *Salame Piemonte* which was characterized by the dominance of a limited number of lactic acid bacteria (LAB), coagulase negative staphylococci (CNS) and fungal species. Sausages from the different batches were different when the volatilome was taken into consideration, and a strong association between quality attributes and LAB strains present was determined. Finally, autochthonous starter cultures were developed and evaluated for sausage preparation at a pilot-scale and promising results were obtained showing the interest of using autochthonous starter cultures to improve product sensorial properties.

**Titre:** Sélection de ferments autochthones pour la production de Salame Piemonte IGP via l'étude de son microbiote

**Mots-clefs** : Saucisses fermentées, Sélection de ferments, Microbiote, Mycobiote, Méthodes culture-dépendantes, Approche métagénomique, Volatilome

La fabrication et les propriétés organoleptiques des produits fermentés artisanaux reposent sur l'activité métabolique de communautés complexes et souvent mal caractérisées. Ce projet de thèse avait pour objectif l'étude de la diversité, de la dynamique et du rôle fonctionnel du microbiote impliqué dans la fabrication d'un produit fermenté méditerranéen, le *Salame Piemonte* IGP. Un autre objectif était de développer de nouveaux ferments composés de souches autochtones pour améliorer la qualité globale des produits. Des méthodes culture-dépendante et –indépendante ont été appliquées sur trois lots de saucisses fermentées de manière spontanée permettant de révéler la diversité bactérienne et fongique et la dynamique microbienne du *Salame Piemonte*. Ce microbiote était dominé par un nombre limité d'espèces de bactéries lactiques (LAB), de staphylocoques à coagulase-négative (CNS) et de champignons. Les saucissons différaient de par leur volatilome et une forte association entre leurs attributs qualité et les souches de bactéries lactiques présentes a été mise en évidence. Enfin, des ferments issus de souches autochtones ont été développés et évalués pour la production de saucisses à l'échelle pilote et des résultats prometteurs ont été obtenus montrant ainsi l'intérêt d'utiliser des ferments autochtones pour améliorer les propriétés organoleptiques des produits.