Alma Mater Studiorum – Università di Bologna

DOTTORATO DI RICERCA IN

SCIENZE E TECNOLOGIE AGRARIE, AMBIENTALI E ALIMENTARI

Ciclo XXXII

Settore Concorsuale: 07/11 Microbiologia agraria

Settore Scientifico Disciplinare: AGR/16 - Microbiologia agraria

Stressful conditions and metabolism of *Lactobacillus sakei*: impact on the characteristics of fermented products

Presentata da: Michael Magnani

Coordinatore Dottorato
Prof. Massimiliano Petracci

Supervisore
Prof. Fausto Gardini

Esame finale anno 2020

Table of contents

CHAPTER 1:

SALAMI

1.1. FERMENTED MEAT PRODUCT: SALAMI

The word “salami” is used to define, in a very general way, fermented meat-based food products, treated and preserved by curing. This definition is also reported in the Decree n. 231 of 21.09.05 of the Italian Ministry of Productive Activities: “Salamis are meat products obtained from striated musculature, belonging to the pig carcass, with the addition of salt and possibly meat from other animal species, minced and mixed with pork fat in varying proportions, and stuffed into natural or artificial casings; dried and ripened in climatic conditions likely to determine, during a gradual reduction of humidity, the evolution of natural fermentation and enzymatic phenomena, with the aim to guarantee preservation and safety".

Salami is typical of the Italian tradition, in fact only in a few other countries (for example Hungary) is a similar product, cylindrical, more or less regular, characterized by a grain (size of the fragments of the meat batter) more or less fine and from the presence of fat that can be minced together with the lean meat or added in the form of lard, in the mixing phase. The consistency of salami is generally firm and compact, even if some fermented sausages rich in fat may have softer consistency. When cut, the salami must have a smooth and homogeneous surface, the colour must be typically red, uniform and interspersed with white fat, which must be well demarcated by the lean. The meat traditionally used derives from pork, considered for this use of greater value, and the current Italian legislation requires the producer to declare on the label the type of meat used (pure pork or mixed pork). A general recipe for a fermented sausage consists in approx. two thirds of lean meat, one third of fat, salt (2.5-3.5%) spices (black pepper, garlic, fennel, etc.) and preservatives (nitrite/nitrate).

The lean meats used to produce salami include the skeletal muscles of pig, but also other animal species can be used, such as bovine or more rarely meat of goat, sheep, horse, donkey, goose or game (wild boar or deer). The amount of lean meat can vary but is generally around 60-70%. The fat is exclusively pork, for organoleptic and preservative reasons, in particular bacon and throat fat, while the dorsal lard and the one coming from the trimming of the thigh is too soft and greasy to be used for this type of product. Sea salt is used in concentrations of 25-35 g / kg of meat batter, however the request of consumers to decrease the sodium intake for health reasons has led different producers to reduce salt concentrations. Sucrose, dextrose and more rarely lactose and fructose, are sometimes added

in concentrations lower than 0.15%, to favor microbial proliferation, contributing to faster acidification and to the development of organoleptic characteristics (Cappelli and Vannucci, 2000).

1.2. BIOCHEMICAL CHANGES DURING RIPENING

During fermentation and ripening, transformations that influence the rheological, organoleptic and qualitative characteristics of salami take place (Figure 1.1.).

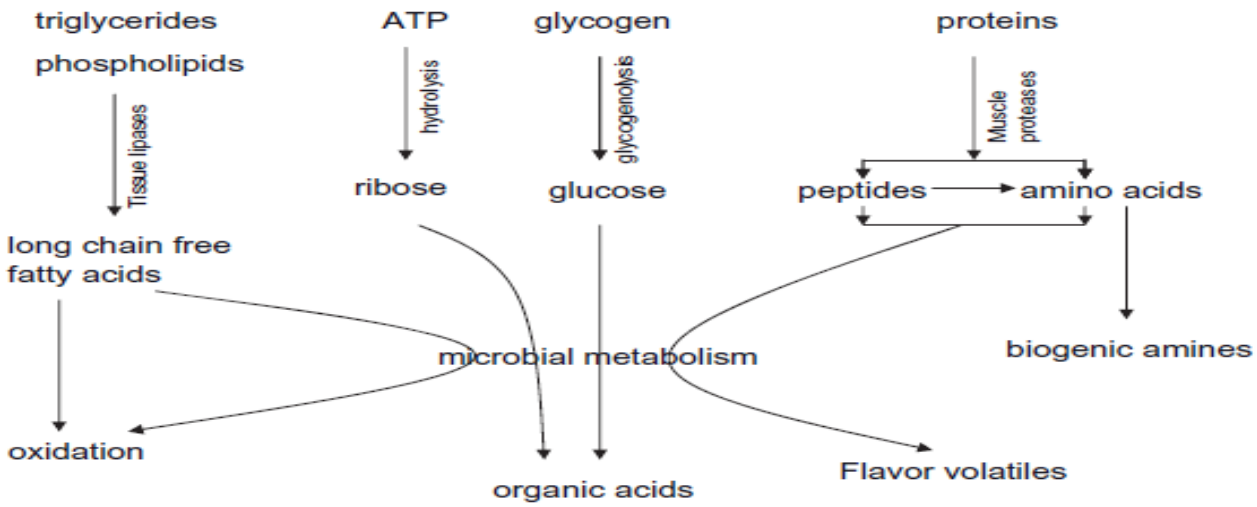

Figure 1.1.: Main biochemical changes occurring during sausage fermentation and ripening.

Microorganisms mainly use carbohydrates as primary energy. The main product of fermentation is lactic acid, which causes the lowering of pH. The residual sugar that remains inside the final product contributes to giving organoleptic characteristics to the salami. This drop in pH causes the inhibition of pathogenic microorganisms eventually present in the matrix. Regarding the proteolytic process, it is mainly carried out by muscle proteinases (Luecke 2000), in particular cathepsin D. The proteolytic capacity of lactic acid bacteria (LAB) isolated from fermented cured meats has been extensively studied, but this ability is reported to be very low (Drosinos et al. 2007). While LAB can hydrolyze mono and triacylglycerol (Sanz et al. 1998), staphylococci can produce lipolytic enzymes able to hydrolyze more complex triglycerides (Casaburi et al. 2007). Once the fatty acids are released, they undergo oxidative reactions that originate alcohols, ketones, aldehydes and esters. If these reactions occur excessively, the fats undergo the rancidity process, which is influenced by the presence of oxygen inside the product.

1.3. THE SPONTANEOUSLY FERMENTED SAUSAGE ECOSYSTEM

The ecosystem of spontaneously fermented cured meats is made by adding simple carbohydrates, spices in a mix of minced meat and fat. The product obtained is inserted into the casings, hung and left to mature spontaneously, allowing the proliferation of the microbial population naturally present in the meat. The different environmental conditions and characteristics of the salami (for example the raw material) induce a different ecosystem within the product, influencing its chemico-physical and sensorial characteristics. In these types of products, the microbial ecosystem is dominated mainly by the microorganisms naturally present in the raw material (Lebert et al. 2007), among which it is easy to find also some pathogens such as *Escherichia coli*, *Staphylococcus aureus* and *Listeria monocytogenes*. During curing, due to the lowering of water activity (A_w) to 0.96-0.97, microorganisms belonging to the group of LAB take over the product (Luecke 1998). In fact, even if the type of raw material used varies, as the seasoning conditions, the microbial ecosystem consists of:

- 10^7-10^9 cfu / g of LAB;
- 10^4-10^6 cfu / g *Micrococcaceae* and enterococci;
- 10^2-10^4 cfu / g of yeasts and molds.

Technological advances and the development of new methodologies for the identification of microorganisms have allowed a deeper study of its composition. One of the first methodologies used is a phenotypic approach. This methodology is based on the study of the characteristics of an organism resulting from the interaction between its genetic constitution and the environment. The most frequently isolated microorganisms are *Lactobacillus curvatus*, *Lactobacillus plantarum*, *Lactobacillus sakei*, *Staphylococcus carnosus*, *Staphylococcus saprophyticus* and *Staphylococcus xylosus*. This is attributed to the fact that these microorganisms are naturally present in the raw material and take over during fermentation.

1.4. MICROBIAL STARTER: COMPETITIVENESS IN MEAT

The choice of the starter cultures to be used in the production of salamis is a crucial point in the production process: it is important to understand the effect they could have on the finale product in order to verify the effectiveness of the selected starter (Hansen 2002). A

fundamental skill is the ability to compete in the early stages of fermentation with the microbiota naturally present in the meat mixture and remain dominant during the entire production process. Furthermore, the starter culture must be able to survive the adverse conditions that occur in the salami mixture (anaerobic atmosphere, high salt concentration, low temperatures and low pH). Among the microorganisms used as starter, *L. sakei* species is the most widely used since it can survive these adverse conditions. In fact, it is able to grow at low temperatures and at concentrations of sodium chloride (NaCl) up to 10%. These characteristics are associated with the presence in its genome of genes that encode proteins that respond to stress conditions, such as cold shocks and osmotolerance. Sanz and Toldrà (2002) report that *L. sakei* has a high activity in the arginine deaminase pathway. Arginine is an essential amino acid for *L. sakei* and being present in the flesh promotes its growth in the environment when the main carbon source (glucose) starts to be scarce (Champomier-Verges et al., 1999). Furthermore, genome analysis has shown that *L. sakei* has a presumed new arginine-deaminase pathway, which can contribute to further increase the metabolism of arginine, thus increasing its competitiveness in the meat environment (Chaillou et al., 2005). These microorganisms, which are partly present in the natural ecosystem of the meat and partly added as starters, become the dominant population during the fermentation process.

Over the past 20 years, the ecosystem of fermented meats has been extensively studied, confirming the knowledge already possessed, but also some new discoveries, especially regarding LAB and coagulase negative cocci (Rantsiou and Cocolin 2006): the most identified species among the LAB are *L. sakei*, *L. curvatus* and *L. plantarum* (Urso et al. 2006a), while among the coagulase negative cocci (CNC) we find *S. xylosus*. The addition of microbial starters (both LAB and CNC) can improve the stability and health safety of the final product, also standardizing the production process (Talon et al. 2008).

An important role in the microbial ecosystem of meat is played by LAB: they can influence both technological and "safety" properties of the product, by reducing the pH following the

Species	Functional and technological properties for meat fermentation	Quality characteristics
Lactobacillus curvatus, *Staphylococcus carnosus*	Fast acidification, and positive mild aroma developments as well as a stable color in the product. The final pH may be adjusted with the amount of fermentable sugars added to the meat mix.	Preservation Firmness (consistency) Aroma
Pediococcus acidilactici, *Pediococcus pentosaceus*,	Create a combination of normal acidification, a positive aroma development, and a good, stable red color in the product. The final pH may be adjusted with the amount of fermentable sugars added to the meat mix.	Preservation Firmness (consistency) Aroma
Staphylococcus xylosus, *Pediococcus pentosaceus*	Acidification process initiates quickly and results in a medium pH-decline. *S. xylosus* gives a strong and stable color and an aromatic flavor.	Preservation Firmness (consistency)
Pediococcus acidilactici, *Lactobacillus curvatus* and *Staphylococcus xylosus*	Fast fermentation, distinct and very good taste, good color formation and stability. Due to bacteriocin production, both *L. curvatus* and *P. acidilactici* contribute to suppressing growth of *Listeria monocytogenes*.	Preservation (pH and bacteriocin) Firmness (consistency) Aroma
Lactobacillus sakei, *Staphylococcus carnosus*	Mild acidification, and positive mild aroma developments as well as a stable color in the product.	Preservation Firmness (consistency) Aroma
Pediococcus pentosaceus, *Staphylococcus carnosus*	Mild acidification, and positive mild aroma developments as well as a stable color in the product*	Preservation Firmness (consistency)
Lactobacillus pentosus, *Staphylococcus carnosus*	Aromatic cultures with intermediate acidification	Color Aroma Preservation
Lactobacillus sakei, *Staphylococcus xylosus* and *Staphylococcus carnosus*	Proteolysis Amino acid catabolism Lipolysis Antioxidant properties: catalase and SOD Nitrate reduction	Color Aroma Preservation
Staphylococcus equorum	Flavor development Nitrate reduction	Color Aroma Preservation
Kocuria varians	Nitrate reduction	Color Preservation

Figure 1.2.: Composition of some commercial starter cultures used for meat fermentation

production of lactic acid and acetic acid. Furthermore, reaching pH values of 4.6-5.9, the muscle proteins coagulate, losing their ability to retain water, providing a better slicing of the final product: it also improves the ability to maintain colour. No less important is the role played by CNC, as they can prevent product rancidity and contribute to the characteristic aroma. In addition, yeasts and molds can be also used as starters, as they can transform lactic acid by producing other characterizing molecules at the level of taste (Lücke 2000). The commercially available starter cultures are generally a mixture of LAB and CNC (Figure 1.2.).

1.5. MICROFLORA ASSOCIATED WITH FERMENTED MEATS

Most LAB used in the production of fermented meats belong to the genus *Lactobacillus*. This genus includes more than 150 species, emphasizing a wide variety of phenotypic characteristics (Axelsson 2004). Among them, we find the *L. sakei*, *L. curvatus* and *L. plantarum*. These microorganisms can degrade the sugar present in the meat mixture, mainly through the way of glycolysis. When hexose sugars (e.g. glucose) are present, lactic acid is the main end product of this process. *L. sakei* is the predominant species in the fermented meat ecosystem (Leroy et al. 2006).

Pediococci, always belonging to the group of LAB, are also used as a microbial starters, although in lesser extent (Papamanoli et al. 2003). These starters are indeed used in fermented meats produced in the United States, where a quick acidification is performed. The genus *Pediococcus* is composed of nine species, but only *Pediococcus pentosaceus* is used as starter cultures.

Other microorganisms very important in the production of fermented meats are those belonging to the genus *Staphylococcus* (*Staphylococcaceae* family). This genus includes 41 widely described species (Garrity et al. 2004). These microorganisms are very common in nature, among which we find the *Staphylococcus xylosus*, *Staphylococcus carnosus*, *Staphylococcus equorum* and *Staphylococcus saprophyticus*. They are facultative anaerobes, able to metabolize different types of sugars to produce lactic acid; the latter can be transformed into acetate, pyruvate and vinegar. These microorganisms have important technological properties, being very competitive in the habitat in which they are found: they are able to survive high salt concentrations and low temperatures during the fermentation process.

1.5.1. FLAVOR FORMATION

The aroma of fermented meat is a combination of several factors. LAB produce lactic acid, acetic acid, ethanol. Another very important factor, which contributes to the sensory impact, is the lipolytic and proteolytic activity carried out by *Staphylococci*. The proteolytic process is influenced by several variables including the ingredients and starter used and the fermentation conditions (Hughes et al., 2002). Staphylococci are able to produce mainly metabolites of pyruvic acid, are able to oxidize fatty acids and methylketones (Stahnke et al., 2002). In particular, *Staphylococcus xylosus* and *Staphylococcus carnosus* are able to metabolize amino acids (leucine, isoleucine and valine), degrading them to branched aldehydes, alcohols and acids (Beck et al., 2002). The presence of *S. carnosus* in the starter culture is able to significantly reduce the maturation time of dry fermented sausages (even 3 weeks) (Stahnke et al., 2002). Microbial proteolytic activity is usually not very high (Kenneally et al., 1999), but may contribute to the initial breakdown of proteins (Fadda et al., 2002). It was found that an endogenous bacterial peptidase could contribute to the final aromatic profile of the final product (Drosinos et al., 2007), in particular several *Lactobacillus* spp. showed proteolytic activity on muscle proteins in pigs. *Lactobacillus*

sakei also plays an important role in the degradation of amino acids (Sanz et al., 1999). Also lipolysis and proteolysis play an important role in the formation of the aroma, which lead to the production of oxidative degradation of fatty acids in alkanes, alkenes and ketones (Chizzolini et al., 1998), which improve the development of taste. Medium and long chain fatty acids are precursors of aromatic compounds (Ansorena et al., 2001). Although these degradations are mainly driven by tissue lipases, some authors (Galgano et al., 2003) attribute some lipolytic enzymes to staphylococci. These starters can contribute to the fermentative process during the production of fermented meat, or they can provide protection to the final product without affecting the organoleptic profile of the product. The ability to produce antimicrobials (such as bacteriocins) can be a very important factor in choosing the starter to be used. Another important role of starters used in meat is also the rapid production of organic acids: these acids are able to inhibit the growth of undesirable microrganisms, increasing the shelf-life of the product.

1.5.2. PRODUCTION OF ORGANIC ACIDS

Sugars (glucose and occasionally lactose or sucrose) are used as ingredients in the fermented meat-based product industry, although some products are obtained without the use of these ingredients (for example Chorizo). During the fermentation process, LAB convert glucose into lactic acid, which is the main cause of the pH decrease. This acidification has a preserving effect, since it induces the inhibition of pathogenic bacteria and, not less important, contributes to the development of the typical organoleptic profile of fermented cured meats (Bover - Cid et al., 2001). The degree of acidification in the final product is influenced also by the choice of the starter culture used in the product. In fact, in Northern Europe, consumers prefer products with a lower pH, which can be obtained using starters such as *Pediococcus* and carbohydrates equal to 0.6% -0.8%. On the contrary, products with a higher pH and lower glucose concentration are preferred in the markets of Southern Europe, using *Staphylococcus* as the dominant starter. The latter requires a longer period of maturation (up to 60 days), and the increase in pH occurs in the successive phases, linked to the metabolism of amino acids and to the production of ATP. The lowering of the pH can also take place following secondary fermentation routes. For example, *Lactobacillus sakei* has enzymes that allow catabolizing the nucleotides (for example inosine and

adenosine). These are abundant in the meat, as opposed to glucose (in a ratio of about 2:1) (Chaillou et al., 2005).

1.5.3. NITRATES REDUCTION

Nitrate is used in fermented cured meats, as well as for its antimicrobial properties, to stabilize the typical color of fermented products: to be effective, nitrate must be reduced to nitrite (Figure 1.3.), and this ability is reported for *Staphylococcus* and *Kocuria*, thanks to the production of nitrate reductase (Talón et al., 2002). These microorganisms can reduce nitrate to nitrite, a very important process for the formation of nitrosomyoglobin, a molecule responsible for the characteristic red color of fermented meat (Mauriello et al., 2004).

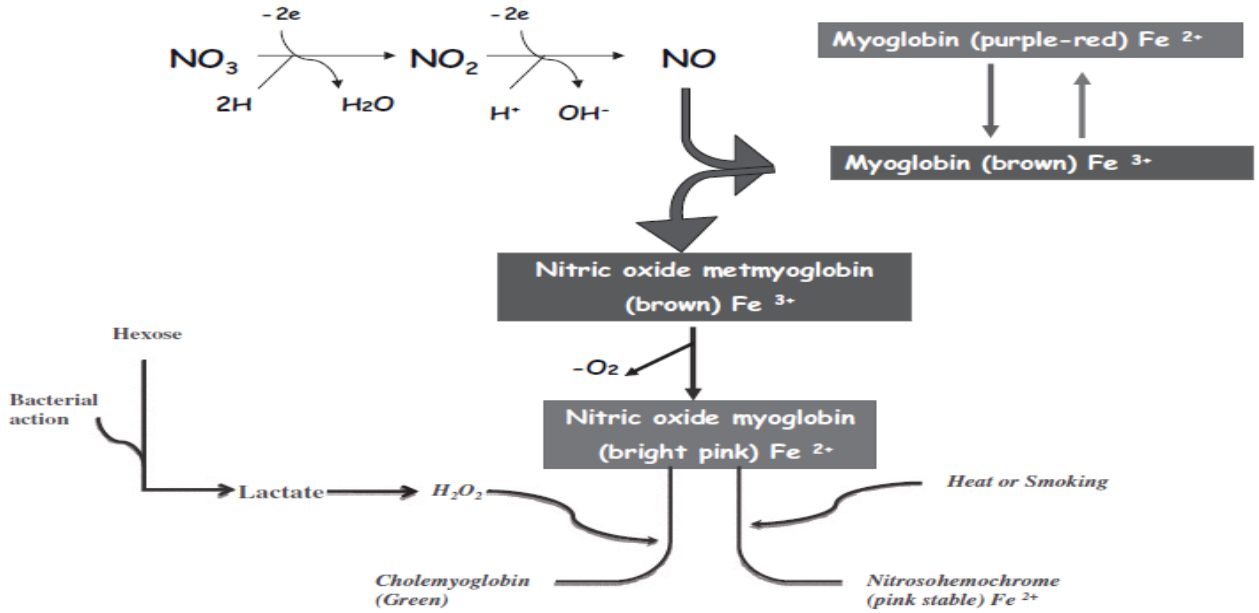

Figura 1.3.: Color development in fermented sausages

1.5.4. CATALASE ACTIVITIES

The metabolism of most LAB leads to the formation of hydrogen peroxide (H_2O_2) which has an effect on the sensory properties of the final product, as it is able to oxidize the lipids naturally present and, by binding to the heme group, it can also cause the product to darken. Several microorganisms used as starters can produce catalase, *i.e.* enzymes that can break down H_2O_2 preventing this risk. This technological property has a relevant aspect in the choice of the starter culture to be used (Leroy et al., 2006). The catalase production capacity has been found in *Staphylococcus carnosus* and *Staphylococcus xylosus* (Barrière et al., 2002), where it has been shown that the transcription of the catalase gene is activated by the presence of hydrogen peroxide. Although LAB are considered negative catalase microorganisms, in the last decade two positive catalase groups have been identified: the genus *Lactobacillus* and the genus *Pediococcus*. Regarding the first group, the analysis of the genome of *Lactobacillus sakei* revealed that it can code a superoxide dismutase to cope with reactive oxygen species. A summary of the main metabolic pathways used by bacteria involved in meat fermentation (mainly *Lactobacillus* and *Staphylococcus*) is described in Figure 1.4.

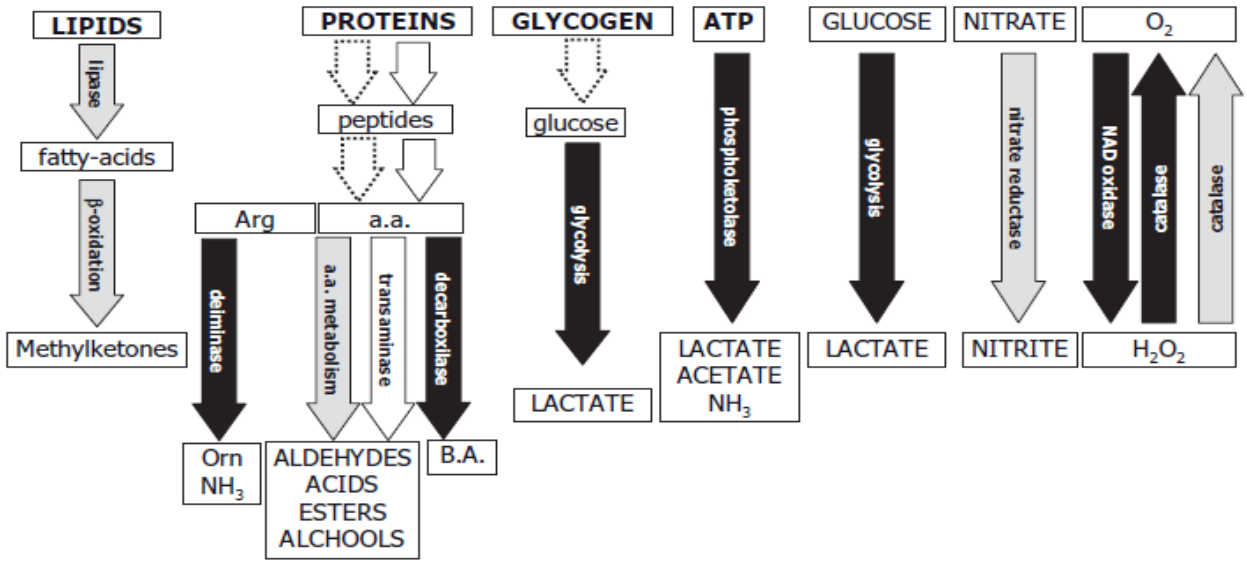

Figura 1.4.: Meat starter culture bacteria: major metabolic pathways in meat fermentation.

1.5.5. PRODUCTION OF BIOGENIC AMINES

The accumulation of biogenic amines in food requires the presence of precursor amino acids and microrganisms capable of degrading them through the use of decarboxylase activity: this reaction is favored by the optimal growth conditions of microrganisms (temperature and

pH). The high quantity of proteins present in meat-based products provides a large amount of precursors for the production of these compounds (Suzzi and Gardini, 2003). The main biogenic amines found are cadaverine, putrescine, spermidine, histamine, phenylethylamine, agmatine and tyramine: they can have toxic effects on the final consumer, such as allergic reactions and poisoning (Shalaby 1996). Attention to these molecules is growing as consumer allergy to them is increasing (Suzzi and Gardini 2003). High levels of tyramine and histamine have been found in fermented sausages (Bover - Cid et al., 2000). Many lactic acid bacteria are able to decarboxylate amino acids to produce biogenic amines. Several studies have shown a strong correlation between pH and biogenic amine content: usually the lower the pH level, the higher the formation of biogenic amines (Parente et al., 2001). The final content of biogenic amines in fermented meats depends on the microbial starter used in the raw material: in fact it was associated with a high production of biogenic amines most of the strains of *L. curvatus* (Pereira et al., 2001). The accumulation of biogenic amines can be reduced by using other microbial starters capable of limiting this accumulation. In the literature it is reported that the use of *L. sakei* CTC494 significantly reduces the accumulation of biogenic amines in the final product (Fernàndez et al., 2003). However, even the use of starter cultures with an amino-oxidasic activity can drastically reduce the quantity of biogenic amines during the fermentation process (Suzzi and Gardini, 2003).

1.5.6. PUBLIC HEALTH ASPECTS

The ability of pathogens (e.g., *Salmonella* spp., *Escherichia coli, Listeria monocytogenes*) to survive in many low - acid as well as low - water activity meat products, such as fermented sausages, makes unlikely to achieve a complete suppression through the application of control measures at a single source (Skandamis and Nychas 2007). Thus, effective control strategies must consider the multiple points at which pathogens can gain access to the human food chain. Measures to control pathogens during fermented meat production, processing, and distribution, at the retail level and during commercial/domestic preparation, should be considered in detail.

Therefore, the best approach to control pathogens in fermented meat products is to implement HACCP principles into the food safety management systems at all stages of meat and meat product production and distribution. However, due to their tolerance to low pH

(i.e., *Listeria monocytogenes*, *Escherichia coli*, and *Salmonella* spp.), some pathogens should be considered at higher risk than other pathogens (e.g., *Staphylococcus aureus*) in meat products (sausages) that have a low pH and are minimally processed or are not cooked before consumption.

1.5.7. PROBIOTICS

Probiotics are live microorganisms that, when administered in adequate quantities, confer a benefit to the health of the host. The use is sanctioned by a regulation of the European Union (EC. n. 1924/2006) which guarantees the use of these probiotic cultures, defining them as safe and scientifically proven. The foods that contain these microorganisms are functional for the consumer and are having a growing popularity in their consumption, being used in a wide variety of foods. Probiotics are often sold as food supplements. Although these probiotic cultures are widely used in the dairy sector, they have been defined as "promising" in the field of fermented meat (Incze 1998). The probiotic starter must be able to develop quickly in the meat and be able to quickly adapt to the environmental conditions that occur, managing to compete with the microorganisms naturally present in the habitat. From most studies regarding the fermentative capacity of these probiotic microorganisms has emerged that they are poorly able to ferment the carbohydrates of meat. This is also confirmed by Erkkil et al. (2001), which state that these microorganisms have poor survival in fermented products such as yogurt, fermented meat and fermented milk. In fermented meat-based products this is due to the various environmental conditions that occur within the meat batter, i.e. the low value of water activity (a_w), the pH and the amount of sodium chloride. Kearney et al. (1990) was the first to suggest the use of microencapsulation with alginate to promote the survival of these cultures in meat. Commercial probiotic starters, such as *Lactobacillus rhamnosus* and *Lactobacillus plantarum* have been tested in Northern Europe for sausage fermentation, but in some case, they affected the sensory properties of the final product (Erkkil et al., 2001).

1.5.8. NUTRITIONAL ASPETCS

The use of microorganisms belonging to the group of LAB can have nutritional effects on food, interacting with the intestinal microbiota and modifying the bioavailability of some nutrients (minerals, vitamins). Meat is a food of high biological value, as it contains micronutrients such as vitamins B1 and B12, minerals such as zinc and iron, easily

assimilated by human beings (Mann 2000). The ingredient used in the production of fermented salamis have been the subject of numerous studies because, due to a high content of saturated fatty acids and cholesterol, they could cause the onset of cardiovascular diseases. Another problem may be related to the high amounts of sodium chloride used, as high consumption can cause high blood pressure. Several substitutes have been examined (Ibanez et al. 1995; Gou et al. 1996; Gimeno et al., 2001) including glycine, calcium chloride, potassium lactate and potassium chloride: besides having found a difference sensory of the product, there is no certainty that it has the same effect from a microbiological point of view. Many alternative products have been studied to reduce the amount of saturated fatty acids in cured meats, with promising results. They foresee the use of dietary fibers (Garcia et al., 2002), soybean oil (Muguerza et al., 2004), inulin (Mendoza et al., 2001).

Since the idea of using probiotic starter cultures in sausage fermentation has developed, several lactic acid bacteria have been screened for their capacity to survive their passage through the human gastrointestinal tract and their possible in - site actions. *Lactobacillus curvatus* RM10 and *Pediococcus acidilactici* P2, isolated from dried commercial meat starter cultures, exhibited the strongest capacity for surviving acidic conditions and 0.30% bile salts (Erkkila and Petaja, 2000). A very effective screening procedure has been suggested by Pennacchia et al. (2004), leading to the isolation of twenty potentially probiotic *Lactobacillus* strains, most of them belonging to the species *Lactobacillus plantarum* (Pennacchia et al., 2006).

1.6. EMERGENCE OF MEAT FERMENTATION AS STRATEGY CONSERVATION

Foods have always been subjects of artistic representations. Already in 2000 b.C. there were frescoes representing culinary subjects (Lindsay, 2012). During the Renaissance, this habit has been growing, reaching its peak in the modern period where it was represented with connotations of opulence or parsimony. In the twentieth century, there was a further huge increase in representation due to globalization (Rousseau, 2012). In addition to the representations, various narratives have been published, often subjected to conflicting

descriptions (Geyzen, 2011). For example, Justus Liebig in 1868 stated that the addition of yeast in bread to carry out the fermentation process deteriorated its nutritional quality (Gratzer, 2008). Another example, more striking, emerged in 1906 when a book was published stating that the consumption of fermented foods caused indigestion (Mathieu, 1906). On the contrary, today the production of fermented foods is highlighted for its beneficial and nutritious characteristics. (Geyzen et al., 2012).

Meat is an extremely perishable product but highly nutritious, and since ancient times it has been a "challenge" to maintain its quality for long periods. The main conservation techniques referred to salting and drying in areas with suitable climatic conditions (Zeuthen, 2007). These techniques induce a reduction in water activity (a_w), resulting in an improvement in the shelf life of the product, and are suitable for intact meat pieces. Conversely, the other cuts (parts of the carcass, fat and chopped meat) need a further fermentation process because of their oxidative and microbial instability. These cuts are usually chopped and stuffed into an animal casing to create a slightly anaerobic environment. Following the fermentation of the meat, some species of LAB are selected from the slightly anaerobic environment, and lactic acid is produced. The salting process, in addition to fermentation and drying, creates a "microbial hurdle" that allows the product to be stored for several months (Ravyts et al., 2012). Furthermore, the lactic acid produced after fermentation contributes to color stability and provides characteristic traits to the final flavor of the product. In countries where drying does not occur sufficiently, due to climatic conditions, a further smoking process was introduced to prevent bacterial deterioration. The first sources documenting the use of these techniques to preserve fermented meats date back to ancient Egypt, although the exact origin is unknown (Pearson and Tauber, 1984). The first written reference dates to ancient Greece, in 600 b.C., but there are descriptions also dated to 160 b.C. from Cato (Zeuthen, 2007). The Romans could have copied this method from the Celts, who used it to preserve meat for the winter months; in fact in their houses the presence of spaces above the hearths allowed the products to undergo the smoking process. Regarding the specific production of fermented sausages, it has Roman origins, which seem to have learnt from Lucan, a tribe of southern Italy. The word "salami" comes from the Latin word "salumen" which means salty product. The Romans established the basic concepts to produce fermented meat, and probably discovered the effect of curing salt

on color maintenance, which was later attributed to the formation of nitrogen monoxide (NO) from nitrite in saltpeter.

1.7. PROPERTIES OF MEAT FERMENTATION IN ADDITION TO CONSERVATION

With the development of the cold chain as the main method of conservation, today the use of the fermentation process for this purpose has become superfluous, especially in Western countries. However, fermented meat products are consumed in large quantities also due to their enormous variety: the fermentation process is considered more as a process of transformation (Ordonez and de la Hoz, 2007). The fermentation process contributes to change the sensory characteristics of the fermented product, as it can produce typical biochemicals compounds (Ravyts et al., 2012). The characteristic consistency is favored by the acidification process, which in addition to the salt concentrations, denatures the proteins, emulsifying the fat present in the mixture. The development of color is due to the interaction between the myoglobin present in the meat and NO (nitrogen monoxide), deriving from the added nitrate/nitrite salts. Finally, the characteristic taste of fermented meats is due to all the chemico-physical processes that take place inside the mixture of salami, thanks to the activity of the enzymes present in the meat and the microorganisms. The aroma profile is indeed influenced not only by the processing conditions and raw materials but also by the microbiota present in the initial mixture (Ravyts et al., 2010). This latter factor plays a significant role in artisanal products, were only the native microflora is present and no starter cultures are added.

Fermented meat-based products are excellent for immediate consumption, easy to transport or store and have a high nutritional value. Thanks to their ease of preservation, they were used as exchange goods and to supply soldiers and expedition campaigns (Zeuthen, 2007). The meats stabilized by this fermentation process have been considered over the history traditional foods. These products have specific ingredients linked to tradition and are processed following old methods and recipes (Guerrero et al., 2009), as illustrated in Figure 1.5.

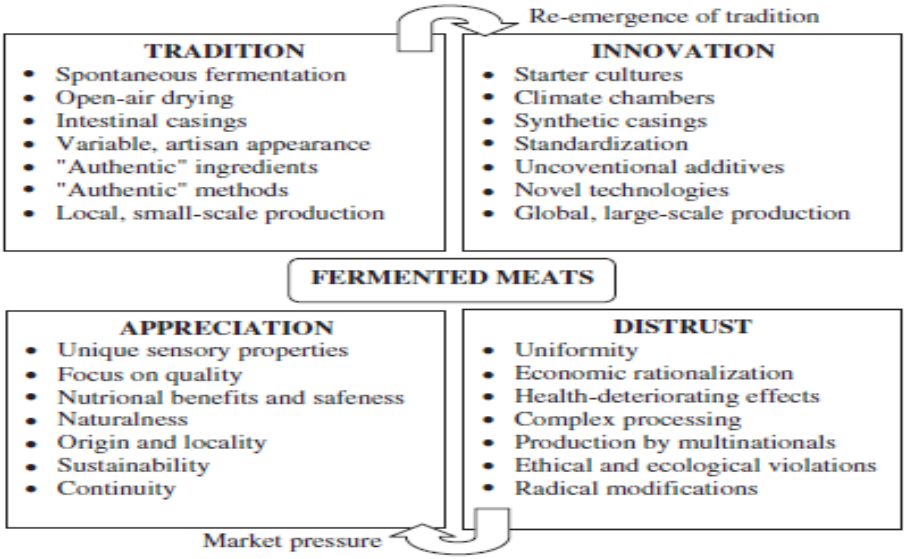

Figure 1.5.: Overview of the contemporary technological and societal positioning of fermented meats in a conflicting framwork of tradition, innovation, appreciation, and distrust, leading to the re-emergence of traditional elements in industrial processes.

The production of fermented meat-based foods has generated a wide variety of recipes and types of regional products, characterized by unique flavors, textures and sensory properties. This differentiation is related to the type and proportion of:

- raw material;
- ingredients;
- sugars and spices;
- type of casing;
- the type of microbiota present;
- fermentation temperature and ripening conditions.

This clearly evidenced that every type of fermented sausage has evolved by virtue of its geographical region: in fact, the place of production has been identified as one of the main characteristics to define a traditional product (Guerrero et al., 2009).

1.8. TECHNOLOGICAL REPRESENTATIONS OF THE QUALITY OF FERMENTED SAUSAGES

Although fermentation is considered as an ancient technology, it has been largely improved, thanks to technological changes, which has led to the evolution of the product itself. This was due not only to the need to improve meat safety, but also to improve the efficiency of the production process. In general, the food sector has transformed rapidly due to the change in the lifestyle of consumers, especially in recent decades (Geyzen et al., 2012). These

changes have created new consumer needs related to product standardization, cost, convenience, etc. In this regard, the production of fermented meat is ideally associated with a rapid and uniform process, thus creating a greater profit margin of the final product (Ordonez and de la Hoz, 2007). This was possible mainly using starter cultures in the meat batter and with the direct use of nitrite instead of nitrate-based salts, but also thanks to the use of higher fermentation temperatures and sometimes even adding acidifiers to speed up the lowering of the pH (Sindelar and Milkowski, 2012). Furthermore, the use of natural casings has been replaced with the more economic and standardized synthetic casings, with impacts on the authenticity of similar salami products. Fermentation rate is one of the most important points of the entire process, since it influences the taste and color of the final product. If the process is performed too quickly it can lead to a serious deterioration of the final quality. When this occurs, some producers try to mask the suboptimal quality by adding additives to improve the taste, including liquid smoke, sodium glutamate, etc… (Roncales, 2007). In recent decades, various opportunities have been implemented by meat industry to improve control over production mechanisms, in order to not compromise the quality of the final product (Leroy et al., 2006). Currently it seems that one of the factors that mainly influences the final quality of the product is the quality of the raw material used. Generally traditional / artisanal products are perceived as superior in quality compared to industrial ones.

1.9. REVALUATION THROUGH TECHNOLOGY AND INVENTED TRADITIONS

Following the emerging negative considerations related to fermented meats, the food industries try to innovate in order to remain competitive on the market (Troy and Kerry, 2011). The boundary between innovation and tradition is always unclear. On the one hand, innovative products are framed in a high legislative and technological context, on the other hand traditional products are considered natural based, and with a low degree of manipulation (Guerrero et al., 2009). A very important point is to succeed in making the consumer understand the concept, apparently contradictory, between innovation and traditional. This very difficult concept is influenced by the country of origin of the final consumer (Guerrero et al., 2009). The image given to the consumer on the product he is

purchasing is fundamental, as regards sensory properties, convenience, product safety, ecological impact and animal welfare (Grunert, et al., 2011). Recent examples include the use of poultry meat to produce cured meats, both to obtain cheaper end products for the final consumer, and to cover a larger part of the market, including Muslim and Jewish populations. However, this has increased the risks associated with pathogenic microorganisms present in poultry (Santchurn and Collignan, 2007). Other innovations include the use of ostrich meat (Bohme et al., 1996), and the use of hazelnut oil as an ingredient (Yildiz-Turp and Serdaroglu, 2008). The main objective of current innovation is to improve the healthiness of food, including the reduction and replacement of salt and nitrites (Zanardi et al., 2010). The technological challenge seeks to reproduce the chemico-physical and sensory properties as similar as possible to those of the original product, but it is not easy at all (Ansorena and Astiasaran, 2007). Other innovations consist simply in the replacement of nitrites and nitrates using some plant extracts that naturally contain the same compounds (Magrinya et al., 2009). Different technological processes have been developed to improve the quality of industrial fermented meat, with attention to the final flavor. Although industrial products are usually cheaper than traditional / artisanal products, the consumer is willing to pay more for these latter products. Traditional products require high quality guarantees and careful labeling, both due to the legislative requirements and to reassure consumers. However, a high presence of details in the labeling could create confusion in the consumer (Grunert and Valli, 2001), so it is important that the labels are very clear as well as being reliable. The European Union, in order to safeguard some food products, has provided means to allow producers to safeguard products, including the use of labels such as:

- DOC (Protected Designation of Origin, PDO), with attention to the origin,

- IGP (Pprotected Geographical Indication, PGI), with attention to production methods.

These labels aim to safeguard the names and characteristics of the products, avoiding imitation and helping consumers in choosing by providing guarantees regarding their characteristics. In Italy, many DOC. and IGP. recognitions have been given, including several products based on fermented meats. This concept is however complex to interpret (Janssens et al., 2012), when the microbiota present in the matrix seems to be associated

mainly with aspects such as the place of production, which also influences the final development of aromas (Ravyts et al., 2012). As a result, the food industry is trying to apply these "spontaneous" starters as innovative and functional cultures for fermented meat products (Leroy et al., 2006).

1.10. METHODOLOGY OF PRESERVATION

1.10.1. DRYING

Drying is probably the oldest method of preservation, together with heating (cooking, frying, etc.), freezing and refrigeration. While freezing and refrigeration were applicable seasonally in the Arctic regions, the drying used alone or in combination with smoking, was practiced all over the world. This method was of vital importance for early humans especially when fishing and hunting was not enough and / or when the flesh and fat of large animals were too much to consume in a short period of time. The unsalted or salted-dried meat was of great importance during wars and sea voyages (Toldrà et al., 2006).

As a result of evolution in general, meat processing began to be done on a large scale, and simple ways of conditioning were elaborated and gradually improved. The well dried meat products were made even before air conditioning. Of course, the process needed conditioning even at that time, but it had other names. The drying chambers had large doors and windows, and air conditioning was regulated by the opening and closing of the doors and windows according to the external temperature and humidity. The water loss of the product was examined by touch, and then possibly based on the color, shape and flexibility of the slices. This procedure needed a lot of experience and was not always accurate.

1.10.1.1. THE BASIS OF DRYING

During drying, the water is removed as vapor. So:

- The activity of microorganisms decreases as the portion of water that can be used decreases, and this in turn leads to an increase in the shelf -life;
- The volume and mass of the product decreases;
- The texture will be harder.

Through these changes, meat products can be processed, stressed and improved to promote the texture of raw meat and develop products with extraordinary attributes.

Bacteria	Yeasts	Molds	a_w
E. coli			0.99
Str. fecalis			0.98
Vib. metschnikovii			0.97
Pse. fluorescens			0.97
Clo. botulinum			0.97
Campylobacter ssp.			0.97
Shighella			0.97
Yersinia enterocolitica			0.97
Clo. perfringens			0.96
Bac. cereus			0.96
Bac. subtilis			0.95
Sal. newport			0.95
Ent. aerogenes			0.94
Microbacterium			0.94
Vib. parahaemolyticus			0.94
Lac. viridescens	*Schizosaccharomyces*	*Rhisopus*	0.93
		Mucor	0.93
	Rodotorula		0.92
Mic. roseus	*Pichia*		0.91
Anaer. Staphylococcus			0.91
Lactobacillus	*Saccharomyces*		0.90
Pediococcus	*Hansenula*		0.90
	Candida	*Asp. niger*	0.88
		Debaryomyces	0.88
	Torulopsis	*Cladosporium*	0.87
Staphylococcus aureus	*Torulaspora*	*Paecilomyces*	0.86
Listeria monocyt.			0.83
		Penicillium	0.80
		Asp. ochraceus	0.80
Halophilic bacteria			0.75
		Asp. glaucus	0.72
		Chrysosporium fastidum	0.70
Zygosaccharomyces rouxii		*Monascus bisporus*	0.60

Table 1.1.: Development of microorganisms correlated to a_w range.

1.10.1.2 THE EFFECT OF CONSERVATION

Vapor pressure of water in foods will be constant in a closed space around the material. This pressure is lower or equal to the vapor pressure of pure water at the same temperature. Its numerical expression is the water activity (a_w):

a_w = vapor pressure of water in the material (foods) / vapor pressure of pure water;

The microorganisms cannot grow below specific aw values (Table 1.1.) (Mossel 1971). The aw of foods/ingredients should be reduced (if possible) beyond a certain level in order to inhibit the growth of contaminating microorganisms.

During drying, this decrease will be achieved by lowering the moisture content. It is necessary to know the relationship between the moisture content and the a_w. This relationship is rather complicated and changes from material to material.

Two groups of components can decrease the value of a_w:

- Water-soluble compounds;
- Materials capable of swelling in water (in meat, structural proteins are such components).

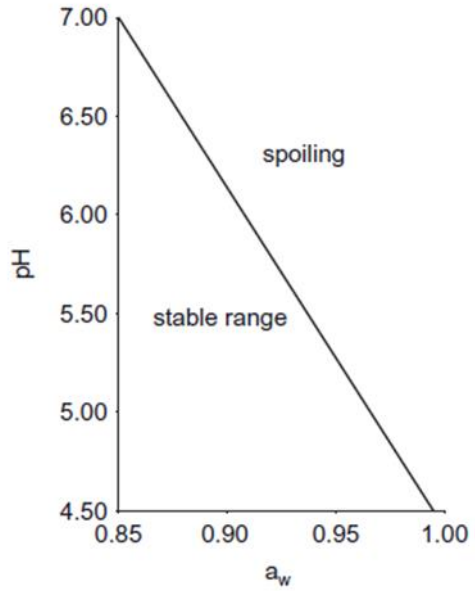

Figure 1.6.: a_w range as a function of pH.

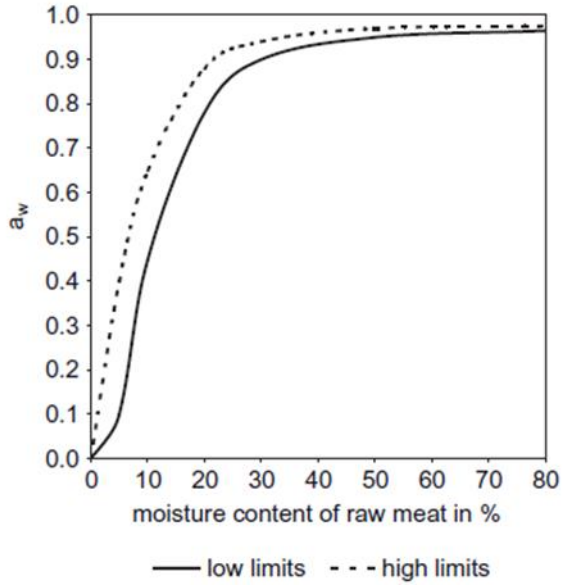

Figure 1.7.: Relationship between aw and moisture of raw meat.

During storage the goal is to decrease the value of a_w below to the desired value. This value depends on the storage temperature of the product. Dry meat products do not need refrigeration; they can be stored at room temperature. Traditional dry meat products have a pH around 6.0; they need a_w less than 0.9. The products obtained through fermentation by LAB have a pH of about 5.0-5.5 and in the latter case an a_w less than 0.95 is necessary to guarantee a safe product (Figure 1.6.) (Incze, 2004).

The Figure 1.7. reports the relationship between a_w and water content calculated in various experiments (Ruiz - Ramirez et al., 2005). The figure also shows that the water content of raw meat must be reduced to 40% to reach a_w value of 0.90.

The meat loses 60% of its original mass during this process. This loss and the amount of energy used is too high. Therefore, raw meat is not dried without having any additional

treatment. Meat powder and meat granules are produced from cooked meat and can be used in powdered soups, soups and foods.

During the reduction of a_w, the following factors must be considered:

- The characteristic composition of the final product (water, meat proteins, salt, other soluble and insoluble components);
- Level of a_w to be reached.

The a_w of meat, which is necessary for stability, depends also on pH. The higher the pH, the lower the a_w should be (Figure 1.8.). The loss of mass and the initial content of salt can be calculated based on the final planned characteristics (Figure 1.9.).

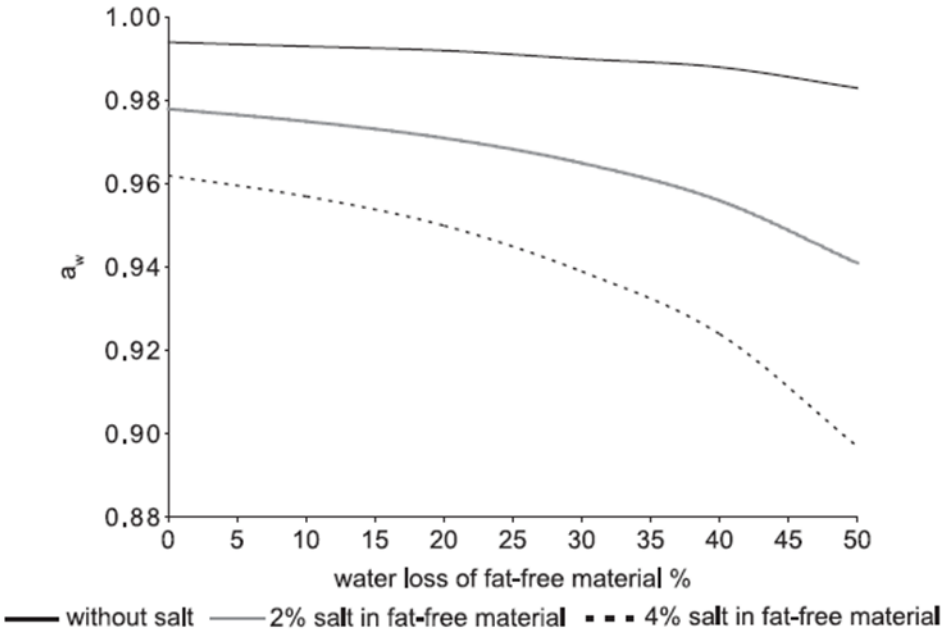

Figure 1.8.: Change of a_w as a function of water loss and initial salt content.

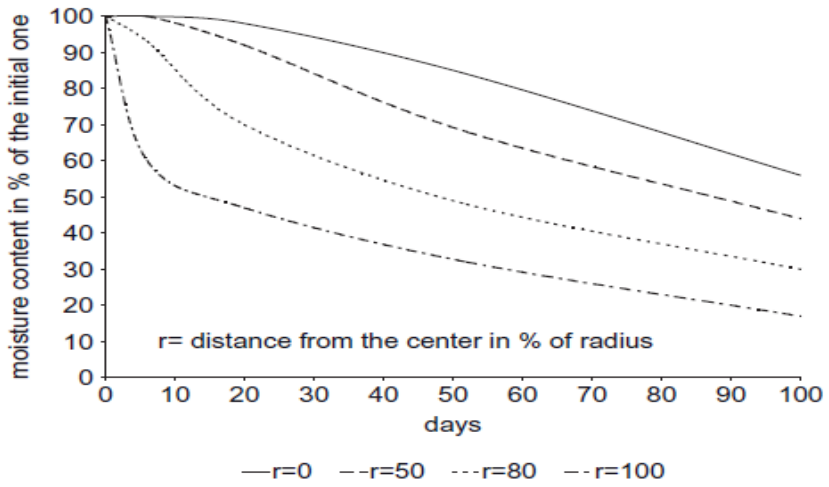

Figure 1.9.: Moisture content in the layers of salami during drying.

1.10.1.3. THE DRYING PROCESS

During drying:

- the vapor evaporates from the surface of the product, and consequently the composition of the surface layer changes.
- The materials move from one layer of the product to the other.
- The thickness (and therefore also the shape) of the layers changes at different degrees.
- An improvement of the mechanical and organoleptic properties of the material occurs.

The water vapor leaves the surface layer of the product if a_w of the surface layer is higher than the air relative humidity in the environment around the product. Otherwise, the surface layer binds the water and gets wet.

The degree of drying depends on:

- The size of the drying surface;
- The difference between the a_w and the humidity of the air (motive force);
- The characteristics of the outer layers with pores, mold, surface layer of the product / resistance to drying).

The drying surface is the geometric surface multiplied by the ratio between the elements permeable to water (meat) and the surface. The ham with the skin dries only on the side of the meat; the side of the skin is isolated from the fat. This barrier effect is used during the maturation of some types of hams. If the surface of the meat is smeared with fat, the maturation process, which produces the aromas, proceeds with a very slow drying. The permeable part of the surface decreases to a greater extent than the geometric surface because the particles of fat are attenuated, occupying a greater part of the surface. The water vapor coming out of the surface increases the moisture content of the air in the environment. The relative vapor content will be greater because evaporation cools the surface and the environment. The air surrounding the product must become drier and warmer to maintain the driving force.

The high-speed air circulation also reduces the drying resistance of the side to air. The strength of this side of the product depends on the porosity of the casing, the mold and the grease present on the surface. This resistance increases constantly during the process, reducing the drying rate.

The salt content of the surface layer increases with the loss of water and decreases with the spread of salt in the inner layers. The driving force of the diffusion is the difference in the salt content with respect to the water content. The salt diffusion seems to be more rapid

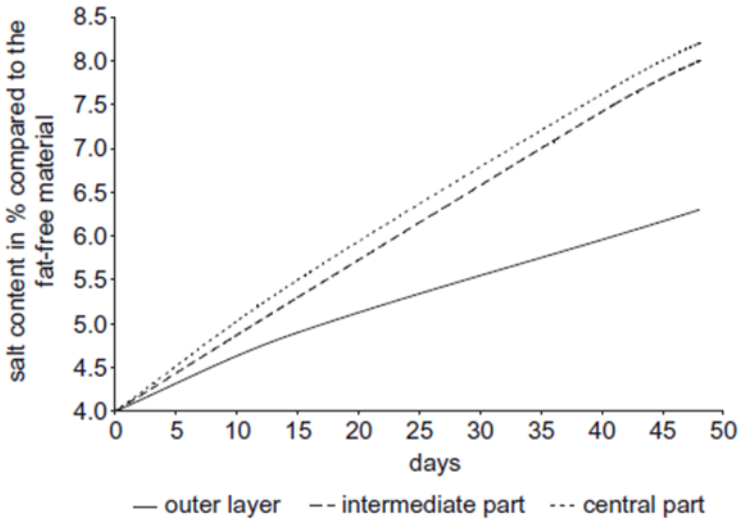

Figure 1.10.: Salt content in the layers of salami during drying.

because the salt content along the product radius is more balanced with respect to the water content. Therefore, the salt content with respect to the water mass is lower in the outer layers (Figure 1.9.) (Gou et al., 2004).

In the initial phase of drying, the moisture content of the outer layers decreases, while the inner layers lose water in subsequent periods. The differences in the water content of the layers change just after the end of drying (Figure 1.10.) (Imre, 1974).

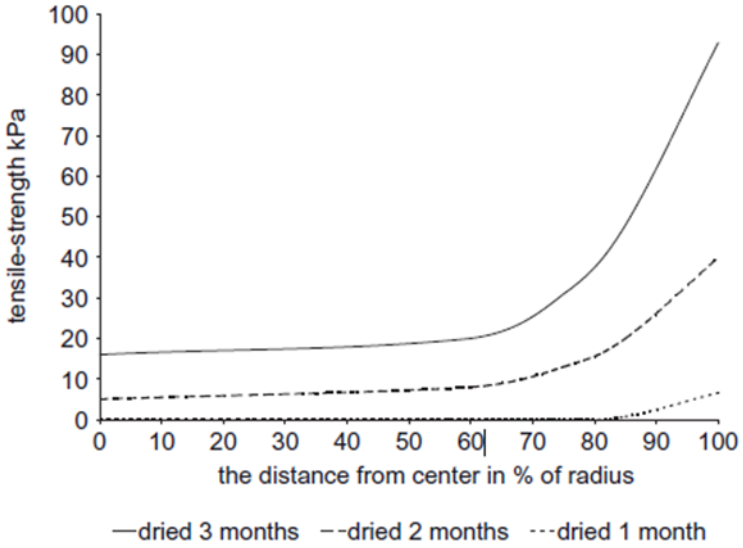

Figure 1.11.: The change of tensile strength of the layers of salami during dryng.

The mechanical properties of dried meat products, such as chewiness, ease of cutting, appropriate stability flexibility, are very important. During drying the initial plasticity disappears and the described attributes increase.

Firmness is highest in the surface layer as a result of the higher content of dry material (Figure 1.11.) (Ruiz-Ramirez et al., 2005). Too low water content in excessively dry products causes protein denaturation and a loss of swelling capacity. The result is a product that is too chewy.

Volume loss causes tensile strength in the outer layer; therefore, first, this layer extends in a plastic way and, subsequently, in an elastic way. Too fast drying causes denaturation of the outer layer, so this becomes irreversibly hard (in the case of hardening). The layer should shrink during further drying but is unable to do this.

As a result of the enzymatic decomposition of proteins, the meat softens during drying / maturation (Toldrá 2006). The soft consistency can be controlled with mild heat treatments (below 50°C) at the end of drying (Morales et al. 2008).

The temperature and humidity and air intake are easy to measure and record at the entrance and exit of the air channels. However, this data provides an overview of the room. This distribution must be periodically examined. Water loss can be measured simply by weighing (possibly continuously) some of the product units in the room.

1.10.2 SMOKING

The smoking, drying and salting of the meat are among the oldest methods of food preservation. Years ago, the meat hung over a fire was preserved by the combined action of drying and smoking, often preceded by marinating in brine. The smoking extends the shelf-life and changes the sensory properties of the meat. Smoking procedures have gradually developed to meet the needs of people in different regions of the world in terms of shelf - life and sensory properties. The role of the conservative effect of smoking has decreased in many countries, while taste and safety are of fundamental importance for transformation and the consumer. Today, various smoking procedures are applied throughout the world for the treatment of meat and sausages for domestic use, as well as in large processing plants for the market. It is estimated that 40-60% of the total meat and meat products are smoked. The meat hung in an oven are exposed to the smoke and heat for enough time to cause sensory and conservative effect. Smoke generally comes from steaming wood shavings or sawdust, placed directly under suspended meat or in an external generator. The intensity, relative humidity and flow rate are controlled in a traditional smoking furnace by natural flow and depend on the construction of the furnace, the environmental conditions and the operator procedures. In modern automatic smokers, the flow is forced by mechanical equipment and modeled according to a computer program adapted to the type of smoked goods. The temperature of the smoke affects the sensory properties and the conservative effect and controls the speed of the process. Cold smoke is used in the range 12-25°C and hot smoke at 23-75°C. In hot smoke, since the thermal denaturation of meat proteins is required, the temperature of the smoke during the various phases varies from about 50°C to 70°C.

In recent decades various aspects of the smoking process of meat have advanced. In traditional smoking, the most significant developments include:

- Control of the composition of the smoke through the use of rational smoke generation procedures;
- Use of engineering principles concerning heat and mass transfer to reduce process times and to control product weight loss;
- Optimization of process parameters to ensure the required sensory properties and safety of smoked products.

1.10.2.1 CHEMICAL COMPOSITION OF SMOKE

The chemical composition of smoke and smoke condensates produced by various types of wood has been completely revised over three decades ago (Tóth and Potthast, 1984). In numerous subsequent publications, the effect of prevailing smoke production conditions in different generators has also been studied.

The wood smoke contains air, water steam, CO_2, CO, and at least other several hundreds of organic compounds at different concentrations.

About 400 of them were uniquely identified by chromatographic and spectral analytical methods. The composition of the smoke depends on the type of wood used for combustion (i.e. mainly from its dryness and from hemicellulose, cellulose, lignin and resins content), as well as the air temperature. The vapor content in the smoke can be related to wood and air humidity. The relative humidity of the smoke varies over a wide range and can be controlled by the operator. Although numerous studies (Borys, 1978; Tóth and Potthast, 1984) have been carried out on the effect of generation parameters on the composition of conservation smoke, it is not yet possible to accurately predict the content of various compounds in the smoke. However, the factors which influence the generation of phenols, aldehydes, ketones, alcohols, acids, esters and hydrocarbons are known. Indeed, the concentrations of these fractions in the smoke, in mg/m^3 of the aerosol or in mg/100 g of wood, differ considerably when different conditions of smoke production were applied. The yield and the overall chemical composition of the smoke depends more on the temperature and on the presence of oxygen than to the humidity and the type of wood.

1.10.2.2. THE MAIN GROUPS OF COMPOUNDS

The phenolic fraction of wood smoke consists of about 250 compounds, 85 of which are identified. Phenols are formed mainly from the pyrolysis and oxidation of lignin at a relatively low temperature (200-400°C), and from cellulose at 700°C. The total content of phenols depends on the type of wood, the temperature, and the density of the smoke analyzed. According to different results, 10 to 200 mg/m^3 may be present; the yield of phenols from 100 g of wood varies from 50 to 500 mg. This fraction includes compounds containing one, two, or three hydroxyl groups bonded to the benzene ring, in addition to alkyl or ether derivatives, as well as those containing other alcoholic, aldehyde, acid and ester groups. However, they differ in water solubility, boiling point, oxidation sensitivity, chemical reactivity, sensory properties and antibacterial activity. Among the phenols identified, those present at the highest concentrations are syringol, guaiacol, pyrocatechol, phenol and their various alkyl derivatives. The highest yield of phenols, guaiacol and siringol and their derivatives, compounds that are essential for the sensory and conservative action of smoke, can be obtained reaching temperature of at least 400-600°C.

Aldehydes and ketones in smoking form a group of about 110 compounds, which also include several aldehydes, alcohols, ketoalcohols and ketoaldehydes. In alder and fir smoke, 28 and 34 carbonyl compounds were identified, respectively (Borys, 1978). The aliphatic and cyclic carbonyl compounds, as well as the furan derivatives, are the products of pyrolytic degradation of cellulose and hemicellulos, while aromatic carbonyls are formed from lignin. The total carbonyl compound content ranges from about 25 to 110 mg / m^3; therefore, it is similar to that of phenols. The carbonyls present in the maximum concentrations are acetaldehyde, formaldehyde and acetone; furthermore, several O-heterocyclic carbonyls were identified in wood smoke (e.g. furan aldehyde and 5-hydroxymethyl-2-furaldehyde).

The wood smoke contains several alcohols, aliphatic and aromatic compounds, including methanol, ethanol, allyl alcohol, amyl-alcohol, benzyl alcohol and 2-phenylethyl alcohol. Methanol can act as a substrate for the formation of formaldehyde and formic acid.

The group of carboxylic acids in wood smoke contains about 30 various components. In the hamlet aliphatic some Authors have identified the following acids: acetic, propionic, isobutyric, butyric, crotonic, isocrotonic, valeric, isovaleric, heptanoic, caprylic and

nonanoic acids (Klossowska, 1979). Among the dicarboxylic acids there are oxalic, malonic, fumaric, maleic and succinic acid. Wood smoke also contains several ketocarboxylic acids. In the esters group, the methyl esters of formic, acetic, butyric and acrylic acids, as well as the ethyl ester of benzoic acid have been identified.

One of the most important groups of smoke constituents contains aliphatic and aromatic hydrocarbons. In the fraction of about 20 aliphatic hydrocarbons, the compound present in the maximum concentration is methane, also known as the product of dry distillation of wood.

Wood smoke also contains a few other chemicals, including NO, NO_2 and NO_3, as well as various heterocyclic compounds, including N-heterocyclic pyrrole, pyrazine and carbazole.

1.10.2.3. SENSORY EFFECT

The desirable sensory properties of smoked products derive from the concomitant action of salting and ripening, pre-drying, smoking and heating, and in some cases even dyeing. The smoke compounds induce color and flavor characteristics and interact with the constituents of the flesh, which results in the creation of other sensory active substances. Interactions with the nitrogen components of meat can lead to some changes in consistency. The intensity of the desirable sensory changes induced by the smoke depends on the type of product; some assortments should acquire only a slight smoky note, while for others, mainly regional products, it is necessary to apply a very intense smoke to satisfy the consumer's typical preferences.

1.10.2.4. COLOR

The color developed on the surface of the products is due to the presence of colored components in smoke and to the reactive compounds interactions with those present in the meat or sausage casings. The role of direct coloring can easily be shown by exposing a sheet of tin foil to smoke in the high-voltage field of an electrostatic apparatus; in two or three minutes, the sheet turns yellow or brown. According to Ruiter (1979), a significant contribution to the formation of the color of smoked products derives from the reactions of the carbonyl compounds, mainly glycolaldehyde and methylglyoxal mainly present in the vapor phase of the smoke, with the amino acids of proteins and non-protein nitrogen compounds. Smoke phenols form stable colors in reactions with proteins under weak

alkaline conditions. The intensity (J) of the color of smoked products is mainly related to the optical density of the smoke (E_0) and to the smoking time (τ):

The value of *k* increases with increasing temperature and smoke speed.

High temperatures favor the development of the dark color, because they increase the

$$J = kE_0\tau$$

concentration of the components during the phase of smoke dispersion and the speed of the reaction's amino carbonyl and the polymerization of the various components. The higher the temperature and surface water activity of small-caliber sausages, within limits set by other technological requirements, the darker will be the color of the sausages. The type of wood used for smoke generation is also important. Smoking with beech, maple, ash, sycamore or lime-tree smoke leads to golden yellow; the yellow-brownish color comes from the smoke of oak, walnut and alder and from the lemon as from the smoke of acacia. Products treated with coniferous wood smoke have a dark color.

1.10.2.5. TASTE AND TASTE

Smoke compounds are the dominant factor directly responsible for the smoked flavor. The smoke of steaming wood generated at 450-550°C is considered the most suitable for giving smoked flavor to meats. The products of thermal decomposition of the cellulose and hemicellulose are the result of the caramelization and are the source of fruit aromas, while phenols generated by the decomposition of lignin contribute to the flavor associated with smoking, burning, spices, vanillin and clover odor. Various fractions of smoke condensation separated by chromatography reveal different flavors, including fruity, like diacetyl, spicy, like protein hydrolyzate or that of freshly baked bread.

1.10.2.6. THE ANTIMICROBIAL ACTIVITY OF SMOKE COMPONENTS

The duration of storage of smoked meats depends on the time and temperature of the heating during the process, the decrease in water activity and the antibacterial and antioxidant activity of the smoke components. Therefore, the preservative effect is related to the effectiveness of hot pasteurization, the loss of water during processing, the concentration of salt, the composition and the amount of smoke deposited in the meat. Various products preserved by maturing and intense smoking can have a shelf life up to several months at

room temperature, while a delicate treatment, applied in the production of some sausage types, results in products that can be stored only a few days in the refrigerator. Smoking the sausages for 30 minutes to reach an inner temperature of 60-76°C can reduce the counts of total aerobic bacteria of about two log cycles; higher temperatures and longer processing times are slightly more effective. The components of the smoke delay the growth of the microflora in cold-preserved frankfurters, so the effect increases with the smoking time. Natural smoke can delay the onset of greening of sausages caused by *Leuconostoc mesenteroides* during storage (Anifantaki et al., 2002).

Numerous smoke compounds (phenols, carboxylic acids and formaldehyde) in concentrations like those of strongly smoked products are effective antimicrobial agents. However, their activity against various microorganisms in different stages of development can be different. Phenols prolong the delay phase of bacterial growth in proportion to their concentration in the product. Therefore, the quantity of smoke components deposited on the meat during the smoking process has a significant influence on the preservative effect. In general, hot smoke reduces the number of viable microorganisms in the products of one or two logarithmic cycles, so the effect increases with increasing processing time and temperature. The addition to minced meat of 8% of liquid smoke can reduce the number of viable *E. coli* O157:H7 cells by two logarithmic cycles after 3 days at 4°C. This result, however, has been shown at a very high concentration of liquid smoke, 8%, while the recommended percentage is 1.5% (Estrada-Muñoz et al., 1998). Several *Staphylococcus epidermidis* strains do not survive commercial hot smoke when injected onto the rainbow trout. In cold smoked salmon, the growth of *Listeria monocytogenes* was inhibited in proportion to the smoking time; 12 hours of smoking reduced the population by three logarithmic cycles. However, well-adapted strains can persist in the smokehouse environment, therefore *Listeria monocytogenes* can often be found in vacuum-packed cold-smoked salmon. The total concentration of smoke constituents, present in non-refrigerated foods vacuum packed and lightly smoked, is not high enough to effectively prevent the formation of toxin by *Clostridium botulinum*.

Generally, the vegetative forms of bacteria are more sensitive to smoke. Molds are remarkably resistant. A large population of molds and yeasts can survive in frankfurter sausages smoked for 30 minutes at an internal temperature of 67°C. Smoke has little effect

on yeast count in the early stages of fermented sausage production; however, in the preserved samples, the yeast population is lower in smoked sausages than in non-smoked controls (Watts and Faulkner, 1954).

1.10.2.7. THE ANTIOXIDANT PROPERTIES OF SMOKE COMPONENTS

The antioxidant effect of smoking was previously noted by observing that lipids in smoked meats and fish were resistant to oxidation (Watts and Faulkner, 1954). Phenols are among the components of smoke that have the highest antioxidant activity; some are more effective than hydroxyanisole butylate (E320) and butylated hydroxytoluene (E321) when applied in similar concentrations.

For the phenols arising from smoke most active belong: pyrogallol, 3-metilp the rocatecolo, 4-metil-p-rocatecol or, pyrocatechol, butylated hydroxytoluene, resorcinol, hydroquinone, α-naphthol, 4-metilguaiaol. The antioxidant properties of the phenolic fraction of wood smoke had already been recognized about 50 years ago (Kurko, 1966). It has been shown that the liquid smoke in concentration of 1.5% effectively delays lipid oxidation in precooked beef patties during 90 days of storage at -15°C (Estrada- Muñoz et al., 1998).

CHAPTER 2

MICROBIAL STARTER

2.1. MICROBIAL STARTER

The addition of desirable microorganisms to meat can have four different purposes:

(1) improving safety (inactivation of pathogens);

(2) improving stability (extending the shelf life by inhibiting the undesirable changes caused by spoilage microorganisms);

(3) provide diversity (modification of the raw material to obtain new sensory properties);

(4) provide health benefits (through positive effects on intestinal flora).

The " starting cultures " are used to modify the sensory properties of the food. In meat fermentations, LAB generally comply the first three purposes of the list while other microorganisms, namely the coagulase negative cocci (*Staphylococcus*, *Kocuria*), yeasts (*Debaryomyces*) and molds (*Penicillium*) normally determine and stabilize the sensory properties desired (purpose 3).

The level to be added to meat depends on the growth potential of organisms present in the product: in most European-type sausage fermentations, for example, about 10^6 UFC/g of LAB cells are added while much higher levels of *Lactobacillus* (10^8 UFC/g) are added to achieve the desired effect (Coffey et al., 1998).

The antagonist cultures that are added only to inhibit pathogens and/or to increase the shelf life (purposes 1 and 2), are called "protective cultures", they modify the sensorial properties of the product as little as possible.

2.2. COMSUMER VS INDUSTRY: PROBLEMS AND NEEDS

Consumers obviously want their meat to be "safe to eat ". Interview data indicate that consumers in Germany have more doubts about the safety of meat than about the safety of other foods (Lücke, 1998). They doubt that animal production is environmentally friendly and that animals are treated appropriately during breeding and transport, and believe that abused animals are more likely to fall ill and that sick animal products are not safe. Therefore, aim 1 (security) is very important both for consumers and for industry and an integrated and transparent approach, that involves the entire chain "from farm to table", is needed.

Stability (scope 2) is important, of course, for those consumers without easy and continuous access to meat and refrigeration capacity. In countries like today's Germany, many

consumers say they want their food to be "fresh", although in practice, many of them buy only once a week and the "expiration date" is of great importance in their purchase decision. However, the industry also has a strong interest in saving on distribution costs.

Food fermentations lead to an enormous diversity of products. This is in the interest of both consumers and industry. Starting cultures can help develop and maintain this diversity, but raw materials and other processing factors are more important.

To reduce costs, the industry has a strong interest in standardizing the properties and shelf life of the product, improving the control of microbial processes and shortening the long aging processes necessary for the formation of aroma profile. These problems are probably the main incentives for the meat industry to use starter cultures. However, within a given brand of product, many consumers also expect a high level of uniformity.

The meat industry is facing trends that create problems in the supply of safe and stable products: consumers tend to live in smaller urban homes and have less knowledge of raw materials and cuisine than in the past. Furthermore, many consumers prefer "low-fat" foods with, consequently, higher pH values and water activity (a_w). Furthermore, consumers claim to want " fresh", "natural" foods and no added preservatives. Thus, there is a growing interest in foods that are both "fresh" and "affordable". A clever combination of "hurdles", including "competitive microflora" can lead to such foods. This approach is described by the phrases "Minimal processing" and "Hurdle technology" (Leistner, 1999).

2.3. ANTAGONISM MECHANISM OF MICROORGANISMS

The main mechanisms by which LAB compete with their competitors are the formation of lactic acid, acetic acid and possibly bacteriocins. Some other metabolites of LAB have been shown to inhibit Gram-negative bacteria in vitro (Laitila et al., 1999) but it is unlikely to be exploited to improve the safety and the meat stability: some are not formed in enough quantities (e.g. reuterina), some interfere with the sensory properties (e.g. diacetyl) and some raise regulatory concerns (e.g. benzoic acid).

2.4. LACTIC ACID AND ACETIC ACID

In most food fermentations the acid lactic or acid acetic produced by LAB, and the resulting decrease in pH, are responsible for the effect of conservation. In meat, the main organic acid formed is lactic acid and only low concentrations of acetic acid are acceptable from a

sensory point of view. However, the antimicrobial effect of acetic acid in meat should not be overlooked because, at the same concentration and pH, it is more effective than lactic acid. The sensitivity to these acids varies between different bacteria and depends on the simultaneous action of other factors such as a_w and nitrite. Therefore, in conditions prevailing in many meat products, even small differences in acid concentrations have an important effect on acid-sensitive microorganisms.

2.5. EFFECTS OF MICROBIAL STARTER ON THE SAFETY AND SHELF-LIFE OF MEAT PRODUCTS

2.5.1. FERMENTED SAUSAGES

The microbial antagonism is used empirically in sausage fermentations in which LAB accumulate lactic acid at levels that inhibit the pathogenic bacteria present in the meat and coagulate the soluble meat proteins, thus reducing the ability to bind water and facilitating product drying. The predominance of LAB is favored by anaerobic conditions, addition of salt and sugars below the initial pH of the mixture (<5.8). Formulations (initial a_w and pH, addition of preserving agents and sugars) and fermentation conditions which prevent the growth of pathogens in various types of fermented sausages have been defined (Lücke, 1998). A rapid drop in pH below 5.3 proved to be important for the inhibition of salmonellae and *Staphylococcus aureus* for products fermented at temperatures above 18°C (Schillinger and Lücke, 1989).

Listeria monocytogenes is generally present in raw meat. Its growth potential during commercial fermentation of sausages is low (Farber et al., 1993), there is no epidemiological evidence for the involvement of fermented sausages in listeriosis outbreaks and the International Commission for microbiological specifications in food (ICMSF) recommends a limit of tolerating once up to 100 cells per gram of *Listeria monocytogenes* in such meats (ICMSF, 1994). The use of LAB that produce active bacteriocins against *Listeria monocytogenes* could be further reduced the levels of this pathogen in fermented sausages by about one or two logarithmic cycles compared to a control to which non-bacteriocinogenic cultures with similar acidity activity had been added. This applies to various types of fermented sausages (Figure 2.1.).

Bacteriocin and in situ formation can also contribute to the dominance of the producer strains on other lactic bacteria during sausage fermentation (Vogel et al., 1993). With appropriately selected producer strains, it may therefore be possible to better control the sensory properties of fermentation and minimize the formation of histamine and other biogenic amines that are formed by some strains of lactic bacteria common in meat (Maijala et al., 1995).

Type of sausage	Bacteriocinogenic strain used (*Lb., Lactobacillus, Ped., Pediococcus*)	Decimal reduction of *Listeria* count compared with bac⁻ control strain
"Fresh" (undried, low-acid)	*Lb. sakei* Lb706	1 (regrowth at pH >6.0)
Dried, German-style	Various *Lb. sakei* and *Lb. curvatus* strains	0–2
Spanish style	Various *Lb. sakei* and *Lb. curvatus* strains	0–2
Italian style	*Lb. plantarum* MCS	Slight effect on survival
US-style	*Ped. acidilactici*	0.5–2.5

[a] *Listeria monocytogenes* (References 1, 3, 4, 6–10 see below), *Listeria innocua* (Ref. 2, 5 see below).

Figure 2.1.: Effect of various bacteriocinogenic lactobacilli on *Listeria*[a] in fermented sausages.

None of the strains of LAB active in sausage fermentation have been found to effectively inhibit *Staphylococcus aureus* in meat by bacteriocins. Therefore, these strains should probably be modified by genetic engineering, with consequent problems relating to safety, licensing and consumer acceptance. Furthermore, such cultures would inhibit also staphylococci which are commonly used for sausage fermentations. Finally, *Staphylococcus aureus* is a health hazard only after growth in a food at levels of about 10^7 CFU/g. This growth can easily be prevented by conventional methods. Therefore, no additional safety factors are required for the inactivation of this organism during normal sausage fermentation. Pathogenic clostridia and bacilli do not grow during the fermentation of the sausage (Lücke, 1998).

2.6. PROTECTIVE STARTER FOR UNFERMENTED MEAT

The decontamination processes that inactivate the spoilage flora does not necessarily make it a safer food (Jay, 1996). Meats that could be made safer with the help of protective cultures include raw, unsalted or semi-processed meats and some pasteurized and perishable products such as sliced vacuum-packed sausages such as Bologna type.

There are three approaches to develop protective cultures for these meats:

1) The first approach involves the selection of psychotropic LAB that produce active bacteriocins against *Listeria monocytogenes* and other unwanted Gram-positive bacteria. (Table 2.1.).
2) The second approach involves the selection of psychotropic LAB that, during the cold preservation of products, produce enough lactic acid to affect the growth of other psychotropic bacteria but do not form compounds with low sensory threshold (Table 2.2.).
3) Finally, mesophilic LAB that are activated quickly in case of abuse of product temperature can be added.

Meat product	Bacteriocinogenic strain used (*Lb., Lactobacillus, Ped., Pediococcus*)	Decimal reduction of *Listeria* count compared with bac⁻ control strain
Cured pasteurized sliced vacuum-packed meats, chilled	*Lb. sakei* Lb706	1–3
	Lb. sakei Lb674	4
	Lb. sakei CTC494	1–1.5
Wieners, stored at 25°C	*Ped. acidilactici* JBL1095	1–3
Raw ground meat, chilled	*Lb. sakei* CTC494	1–2
Raw chicken breast, chilled	*Lb. sakei* CTC494	1.5–3
Cooked unsalted meat, chilled	*Lb. sakei* Lb706	1 (regrowth after 7 days)

Table 2.1.: Effect of various bacteriocinogenic strains on *Listeria* in perishable, unfermented

Product	Storage conditions	Target organism(s)	Effect (compared to control)
Ground beef	Vacuum, 4°C	*Listeria monocytogenes*	2 log reduction
Bologna-type sausage	Vacuum, 9°C	*Listeria monocytogenes*	1–2 log reduction
Bacon cubes	10% CO_2, 90% N_2, 2 or 15°C	*Listeria monocytogenes*	0.5–2 log reduction
British fresh sausages	Uncooked, vacuum, 5°C	*Brochothrix thermosphacta*	1–1.5 log reduction
Cooked ham	Vacuum, 4°C	Spoilage flora	Extension of shelf life (7 days)
Frankfurter-type sausages	Vacuum, 6–8°C	Spoilage flora	Extension of shelf life (18 days)
Frankfurter-type sausages	Vacuum, 6°C	Slime-forming *Lactobacillus sakei*	No inhibition

Table 2.2.: Effect of a commercial protective culture on microbial quality of some perishable meats.

2.7. EFFECT OF STARTER CULTURE ON THE SENSORY PROPERTIES OF FERMENTED MEATS

A large variety of compounds is likely to contribute to the desired flavor and aroma of fermented sausages (Dainty and Blom, 1995). Some are added to the sausage as ingredients (salt, spices, smoke components), others are formed by abiotic reactions, microbial tissues or enzymes during maturation. There is great commercial interest in accelerating the ripening processes and prolonging the shelf life (Lücke et al., 1990). The role of bacteria in flavor development has recently been summarized by Flores et al., (2018). The main

products of carbohydrate fermentation by LAB (lactic acid with small amounts of acetic acid) give the sausages an "acid" flavor that predominates in the semi-dry products that are sold after less than two weeks of maturation. The intensity of this aroma depends on the pH value, but at a given pH, a high percentage of acetic acid gives the product a less "pure" and "acid" flavor. High levels of acetic acid accumulate if glucan- δ- lactone (GdL) is added as acidifying and because this compound is fermented by many lactobacilli in lactic and acetic acid. In non-dried products, the formation of acids is limited in order to keep them spreadable and to preserve the aroma and taste of fresh meat.

Lipids and nitrogen-containing compounds are precursors of most of these substances. Tissue enzymes are the main agents of lipolysis (Molly et al., 1996) and proteolysis (Demeyer, 1992), at least in sausages without superficial mold.

Cathepsin D is activated at pH values around 5.0 and produces peptides that are further metabolized by the ripening flora. As maturation continues, bacterial enzymes can also play a role in the degradation of the formed peptides (Molly et al., 1997).

However, this may be due to an indirect effect such as stimulating microbial activity. CNC can influence the aroma and taste of fermented sausages by transforming compounds originating from lipid (non-microbial) and protein degradation into compounds that add the desired aroma to sausages.

Some fermented sausages and raw salted meats - those produced in France, Spain and Italy - are characterized by the development of a superficial flora consisting of molds and yeasts that contribute to the desired sensory properties of the product. Lactate oxidation and proteolysis lead to a noticeably different taste than smoked sausages. As mold growth increases the surface pH of the sausage, it is important that it starts only after the pH and activity of the sausage water is low enough to prevent the superficial growth of unwanted bacteria such as *Listeria* spp. (Rödel et al., 1993). A suitable surface starter should quickly colonize and adhere to the surface of the sausage. This allows it to suppress unwanted molds and protect the product from the damaging effects of oxygen, facilitate drying by "dabbing" the fluctuations of humidity in the ripening chamber and make the desired changes in appearance (whitish or yellowish) and in flavor. Of course, it must not form mycotoxins. Varieties that have been selected are now available

as starters for these products. Most of them contain *Penicillium nalgiovense*, sometimes combined with the yeast *Debaryomyces hansenii* (or its imperfect form, *Candida famata*).

2.8. PROBIOTIC MEAT PRODUCTS?

Information on probiotic bacteria has been summarized by Holzapfel et al., (1998). As pointed out by Hammes and Haller (1998), a significant metabolic activity of probiotic strains in the large intestine would require a daily intake of 10^8 or more cells.

From this it can be concluded that a food labeled "probiotic" should contain no less than 10^6 CFU/g probiotic bacteria unless the manufacturer provides proof that the health claims on the label do not mislead the consumer. A "probiotic" fermented sausage produced with the addition of bifidobacteria has long been marketed in Germany, but the strain used was not able to well survive during the maturation of the sausages and a very high inoculation had to be added to reach at least 10^6 CFU/g of bifidobacteria after fermentation.

Mesophilic lactobacilli are much better candidates for use as probiotic meat cultures, as shown by Andersen (1998). Screening programs can lead to strains that survive both meat fermentation and passage through the stomach and small intestine (Hammes and Haller, 1998) and that can later be tested for their health benefits. In Germany, it is not easy to market probiotic meats because, unlike dairy products, meat does not have the reputation of being a "healthy food", but this could be different in other countries.

2.9. PERSPECTIVES AND LIMITS OF THE USE OF GENETICALLY MODIFIED CULTURES

Tools are available to modify the genome of various microorganisms suitable as starter and protective cultures for the meat. The research has focused on the transfer of individual genes coding for the bacteriocins (Allison et al., 1995) or lytic enzymes such as lysostafine.

The gene for the latter compound was expressed in *Penicillium nalgiovense* (Geisen, 1993) and in *Lactobacillus curvatus* (Gaier et al., 1992). It was subsequently shown that this lactobacillus produces lysostafine in enough quantity to inactivate *Staphylococcus aureus* during sausage fermentation (Cavadini et al., 1998). By transferring genes that code for antagonist proteins and increasing the expression rate of these genes, it could be possible to increase the biocontrol potential of a host strain suitable even for low cellular densities. However, as indicated above, the possible benefit of this approach is very limited

and most likely limited to improving the control of *Listeria monocytogenes* on such meats. It may also become possible to design metabolic pathways in order to give a better control of the speed and extent of the formation of lactic and acetic acids and eliminate undesirable properties such as the formation of biogenic amines. However, these latter goals - with better acceptance by the public and regulatory authorities - can be more easily achieved by selecting the strains from nature.

2.10 STARTER CULTURES

Starter cultures are preparations that contain microorganisms with active growth or whose metabolic activity (Figure 2.2.) imparts the desired effects during fermentation (Hammes and Hertel, 1998). The industrialized production of starter cultures is a consequence of the gradual shift of sausage production from small local producers to large-scale processing plants and the growing awareness of risks to consumer health, in view of the overall efficiency of the process (Magistà et al., 2017). The introduction of starter cultures has become essential to shorten the maturation period, ensure color development, improve flavor and product safety, given that industrial production of fermented cured meats is increasing (Lücke, 1986). In fact, a starter culture should be able to conduct fermentation, colonize the product and dominate other microorganisms from the beginning to the end of the process (Cocolin et al., 2006).

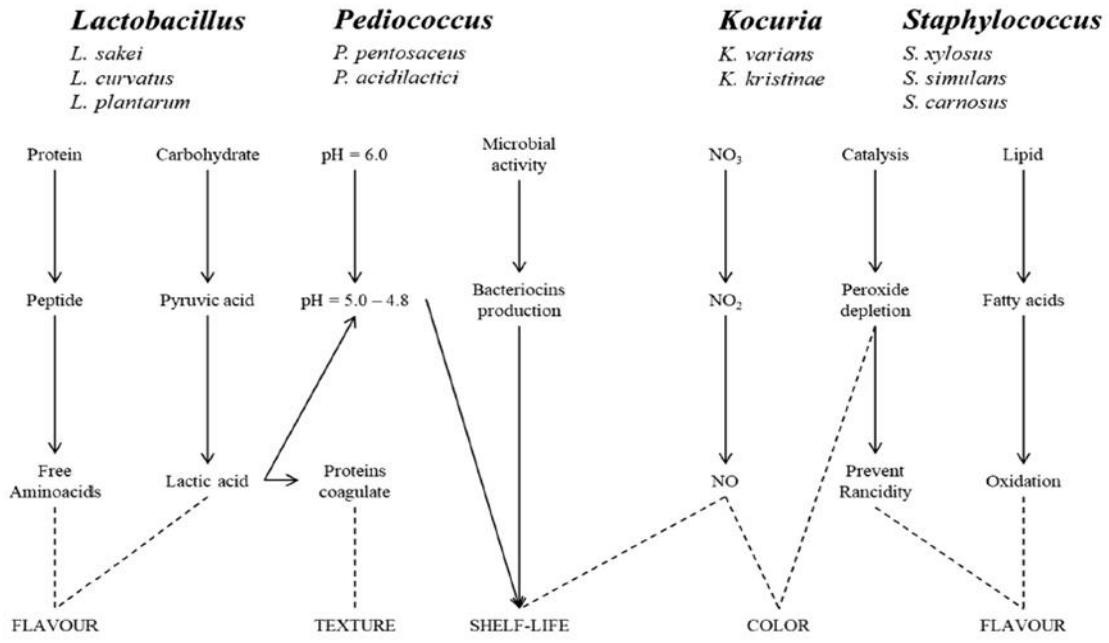

Figure 2.2.: Summary of biochemical activities performed by principal microbial group in fermented sausages (Cocolin et al., 2006)

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

Table 2.3.: Main differences between traditional and industrial fermented food products.

On the other hand, the use of commercially available starters, consisting mainly of LAB and coagulase-negative cocci, can lead to a loss of peculiar organoleptic characteristics present in spontaneously fermented sausages with a depletion of flavor and aroma. The main differences between traditional and industrial fermented product are summarized in Table 2.3.

This is due to the technology used, to the properties of the raw material and to the specific composition of the microbiota (Leroy et al., 2006). However, Sunesen and Stahnke (2003) reported that sausages produced with commercial molds show a more uniform taste, texture, drying speed and a more uniform appearance compared to artisanal sausages.

The microbial ecology of fermented sausages has become of increasing interest in recent decades, given that different genera, species and even strains have been shown to significantly influence the sensory traits of fermented sausages (Rantsiou and Cocolin, 2006). The production of artisanal sausages depends largely on the skill and experience of the meat producer and can be considered an art rather than a process entirely based on scientific and technological knowledge. The fermentation of meat is, in fact, a complex biological phenomenon accelerated by the desirable action of some microbes in the presence of a variety of species with synergistic or competing action. A great variability in the quality of the products is due to the traditional practices and to the variation of the micro-organisms involved in the process. De Vuyst (2000) underlined that it is of primary importance to investigate and analyze the influence of the environment on the performance of an initial culture before its use in a selected product. In order to protect the traditional aspects of these products and select native cultures to be used, it is essential to understand the microbial dynamics during fermentation (Rantsiou and Cocolin, 2006). In Italy,

only *Lactobacillus, Pediococcus, Micrococcus, Debaryomyces* and *Staphylococcus xylosus, Staphylococcus simulans* and *Staphylococcus carnosus* are authorized as starting cultures (Gazzetta Ufficiale, 1995).

2.11. PRODUCTION OF BIOGENIC AMINES

2.11.1. WHAT ARE BIOGENIC AMINES?

Biogenic amines are low molecular weight organic bases produced by free amino acids decarboxylation (Shalaby, 1996) due to the specific enzyme decarboxylase, which is present in some microbial strains (Silla Santos, 1996). Their production of biogenic amines in foods, therefore, requires a microbial population equipped with these decarboxylase enzymes, a substrate rich in precursors and conditions suitable for the development of this microflora within the food system (ten Brink et al.,1990). The chemical structure of biogenic amines can be aliphatic (this is the case of putrescine, cadaverine, spermine and spermidine), heterocyclic (like histamine and tryptamine) or aromatic (such as tyramine and 2-phenylethylamine). Biogenic amines have different toxicological implications if taken in high doses, in fact, limited quantities are not harmful because they are eliminated by special detoxification systems present in human organism. For this reason it is necessary to distinguish them from amines found as natural constituents in many food products (such as vegetables, meat, fish, chocolate and milk) since these generally lack toxic effects. Biogenic amines are generally present in low quantities (even below the determination limit) in fresh foods; therefore, their measurement is necessary/useful because their value increases in poor hygiene, temperature abuse or poor storage conditions. Indeed, the biogenic amine content increases with time and temperature of conservation (Halasz et al., 1994).

The microorganisms capable of producing biogenic amines belong to many genera and species but all are united by the presence of amino decarboxylases. *Bacillus* (Rodriguez-Jerez et al., 1994), *Pseudomonas* (Tiecco et al., 1986), *Photobacterium* (Jorgensen et al., 2000), as well as different genera of the families of the *Enterobacteriaceae*, such as *Citrobacter, Klebsiella*, *Escherichia*, *Salmonella* and *Shighella* (Marino et al., 2000) and of the *Micrococcaceae*, such as *Staphylococcus*, *Micrococcus* and *Kokuria* (Martuscelli et al., 2000) are the main microorganisms able to decarboxylate amino acids. Also, some genera belonging to the group of LAB showed an amino decarboxylase capacity (Landete et al.,

2008), mainly those belonging to the genera: *Lactobacillus*, *Enterococcus*, *Carnobacterium*, *Pediococcus*, *Lactococcus* and *Leuconostoc* (Lonvaud – Funel, 2001). Many of these species are found in foods, but they are also used as a starter to produce fermented foods.

2.11.2. WHY ARE THEY PRODUCED?

The physiological roles of amine formation by microorganisms are not completely known yet. However, it is known that biogenic amines play a role fundamental in the development and physiology of eukaryotic cells (Igarashi et al., 2001). Putrescine, spermine and spermidine regulate the genetic expression of cell differentiation and growth, while among the most active biogenic amines we find histamine and tyramine. Within the microbial cell, one of main causes leading to the decarboxylation process is the need to get energy. This aspect assumes greater importance especially in nutritionally poor environments since the decarboxylase system can generate a translocation of charges through the cytoplasmic membrane that modifies its potential with energy production for the cell (Konings et al., 1997). It is also known that some amines perform important functions in living cells as indispensable and/or regulatory components of function of nucleic acids, protein synthesis and probably also of membrane stabilization, as well as precursors of the synthesis of hormone, alkaloids, proteins and nucleic acids (Silla Santos, 1996). During the first stages of fermentation, the high content of nutritional compounds does not lead to a marked production of biogenic amines since the cell has no difficulty in finding nitrogen-based compounds or carbon sources (Konings et al., 1997). It is therefore evident that these compounds are produced in response to a lack of energy sources or to a stress that significantly affects cellular activity (Konings et al., 1997). As previously mentioned, therefore, the main cytoplasmic membrane is responsible for all those processes that subsequently lead to the formation of amines biogenic as it constitutes a physical barrier to the external environment and absolves two important functions:

- Preserves cellular integrity by containing the cytoplasm, preventing thus the uncontrolled spread of metabolites and cellular ions;
- Transduce chemo-osmotic energy.

The possibility, therefore, of generating the potential necessary to establish an ionic gradient lies in the characteristic limited permeability of the cell membrane. The membrane is

equipped with proton pumps able to create a gradient which is, from the energetic point of view, the most important one (Mitchell, 1996). The pump proton present in aerobic bacteria is represented by the respiratory chain, in which it is has an electron transfer matched to one of protons that are ejected from the cell. In this way two gradients are formed:

1. A pH gradient, as an alkaline and acidic environment is established outside for proton accumulation;
2. A membrane potential generated by the translocation of protons from the inside to the outside.

The complex of these two gradients represents the proton motive force that regulates the flow of protons through which energy is generated and is a typical mechanism of production of ATP via the ATPasi system. The ATP produced is necessary for the cell for the synthesis of cellular constituents, for the maintenance of other energy processes in the cytoplasm and in the cytoplasmic membrane and for the generation of the proton motive force (Maloney,1977). Growth and cell development require the absorption of solutes and precursors of important cellular molecules, but also the excretion of metabolism end products. This expulsion can take place through secondary transport mechanisms, as an alternative to primaries, which, in turn, can generate a driving proton force. For microorganisms this is a fundamental point since their growth substrate is subject to continuous growth changes in composition, and consequently are often subjected to moments of nutritional abundance alternated with moments of deficiency and stress. When they run out energy resources, the rate of glycolysis decreases rapidly and with it also the generation of the proton driving force. It is evident, therefore, that in these periods the bacteria benefit from an alternative metabolic energy supply. There are three main secondary systems of power generation, which are:

- *Energy generated by a single-port system*: it is the simplest mechanism through which the transport of solutes can contribute to an increase in metabolic energy. It consists in the absorption and rapid metabolization of a negatively charged solute or in the expulsion of a final product positively charged. This charge translocation, coupled with the transport of solute or catabolite, generates a potential difference that will give rise to the so-called proton force.

- *Energy generated by the exchange precursor / final product (anti-port):* during the fermentation solutes (precursors) are absorbed, metabolized and expelled as products finals. When the final products are structurally like the precursors, the former can be removed from the cell with the same transport system used to store the precursors. This opportunity is energetically favorable to the cell as the process of transport is guided both by the concentration gradient of the precursor and by that of the final products. Decarboxylation processes of amino acids, with formation of biogenic amines, belong to these energy production mechanisms. These processes are the same for the different amines and for all the known amines. It can therefore be said that the formation of amines has an energetic reason, since they represent one of the many secondary methods of energy production. Some recent studies suggest new interesting hypotheses on the physiological role of amines in microorganisms, such as the important role of putrescine in the expression of microbial genes, particularly those operating in *Escherichia coli* due to stress oxidative, responsible for protecting the cell against hydrogen peroxide and the superoxide radical (Tkachenko et al., 2001). Also, it has been observed that some strains able to decarboxylate amino acids can overwhelm or reduce the effects of temperature, sodium chloride and other biological and physico-chemical factors that induce stress responses from the cell with consequent production of biogenic amines. The polyamines, as shown, can act as endogenous permeability modulators of membrane and probably are part of a mechanism of adaptation to acidic conditions (Samartzidou and Delcour 1999). Biogenic amines are also very important in higher organisms. In fact, it has been noted that in growing tissues they are produced in rather high concentrations compared to a non-multiplying tissue (Bardòcz, 1989). As a consequence, the cells of living organisms can produce such compounds, that are however often taken through the diet. The polyamines, for example, are found in embryonic tissues and in regenerating liver and are essential to maintain a high level of metabolic activity in the normal functions of the intestine and his immune system. They are probably also very important in stabilization of the cell membrane and have an antioxidant effect for polyunsaturated fatty acids (thanks to their numerous amino groups). Spermine and spermidine appear to be involved in the evolution of intestinal tissue,

catecholamines (hormones), indolamines (e.g. serotonin) and histamine play important metabolic functions for the nervous system. Regarding the control of blood pressure, phenylethylamine and tyramine increase it, while in contrast histamine reduces it. From a technological point of view, it should also be mentioned that biogenic amines are precursors of hormones, alkaloids, nucleic acids and proteins; they are also very important in the formation of the aroma of certain foods (Shahidi et al., 1994).

2.11.3. BIOGENIC AMINES IN FOOD

Biogenic amines are present in a wide range of food products including fish, meat, dairy products, wine, beer, vegetables, fruit, hazelnuts and chocolate (Brink et al., 1990).

2.11.4. UNFERMENTED FOODS

In unfermented products, the presence of biogenic amines, beyond certain levels, is considered as an indicator of unwanted microbial activity, even if their presence is not necessarily correlated with the development of degradative microorganisms as not all of them have decarboxylase-positive activity (Vidal et al., 1990).

Biogenic amines can be found in:

- Fish products: mainly in fish belonging to the Sgombroid family which are commonly associated with cases of histamine poisoning. The formation of this amine in fish is attributed to the action of microorganisms with decarboxylase activity against histidine, which is present in large quantities in these products (Halaz et al., 1994). Some different amines, like putrescine, cadaverine, tyramine, spermine and spermidine, have been found in fish species, while others, such as trimethylamine and dimethylamine, are present in relation to fish freshness (Kolodziejska et al., 1994).
- Fruit, juices and vegetables: numerous orange-based juices, nectars and lemonades, lemon, grape, raspberry, mandarin and strawberry contain varying concentrations of amines biogenic and putrescine seems to be present with greater quantity. Halasz et al. (1994) report high levels of amines in orange juices (norepinephrine, tryptamine), tomato (tyramine, tryptamine, histamine), banana (tyramine, tryptamine, serotonin) and spinach (histamine).

- Meat: high levels of biogenic amines (spermine and spermidine) are contained in pork, both fresh and by-products, (Halasz et al., 1994). Histamine and factors favoring its synthesis has been identified in beef, ovine (Teodorovic et al., 1994) and in other types of meat or meat-based products (Santos et al., 1985). Starting from high quality meat, and using good production practices, the risk of formation of biogenic amines is greatly reduced (Hernandez-Jover et al., 1996). In fact, the analysis of biogenic amines is proposed as an index of the quality of fresh meat (Vinci and Antonelli, 2002). The major recorded cases of tyramine in fresh meat have been found in meat preserved beyond the recommended date of consumption (Boulton et al., 1970). The new forms of packaging of fresh meat have led to greater preservability, but this does not prevent the formation of biogenic amines (Krizek et al., 1995). Indeed, while the recommended storage time for the fresh poultry meat is lower than for red meat, many reports suggest that chicken meat products may undergo less changes if properly treated and preserved compared to red meat products (Vinci and Antonelli, 2002). Red meat (especially bovine) was found to have a low content of biogenic amines up to the ninth day of conservation, after which the levels rise sharply and continue to rise, while for the chicken one, strong increases were detected already at the fourth day of storage; this seems to have been attributed to the shorter fibers present in the white flesh of the chicken (Vinci and Antonelli, 2002).
- Milk: in human milk amines such as spermine, spermidine and putrescine were found in varying quantities. In whole or partially skimmed cow's milk low quantities of polyamines have been found (Bardocz et al., 1993).

2.11.5. FERMENTED FOODS

During the preparation of fermented food, it is necessary to take into account the broad variety of microorganisms that can intervene, and which could be able to produce biogenic amines. Many products are obtained by fermentation by LAB with the production of putrescine, cadaverine, histamine and tyramine (Brink et al.,1990), but it has been shown that in these products, the content of these substances is subjected to fluctuations due to the qualitative-quantitative composition of the microflora (which evolves constantly during

fermentation and seasoning), to the chemical-physical variables of the product, to the hygienic procedures adopted and above all to the availability of precursors.

- Cheese and dairy products: after fish, cheese is the food most commonly associated with histamine poisoning, with the first case dating back to 1967 in Holland (Stratton et al., 1991). Numerous studies have been conducted to determine the amine content in dairy products. Histamine, tyramine, cadaverine, putrescine, tryptamine and phenylethylamine have been found in numerous types of cheese. The levels of histamine and tyramine vary according to the different types of cheese, to the process of production, fermentation and maturing (Stratton et al., 1991). The tyramine is clearly prevalent compared to the others, while the histamine would be the fourth most amine present, but in general the quantities are so low that do not make it dangerous. A high concentration of these biogenic amines was detected in mature hard pasta cheeses, made from raw milk. The production of amines can in fact be associated with different parameters
  - Bacterial cell load in raw milk;
  - Use of raw or pasteurized milk;
  - Intensity of heat treatment and its duration;
  - Environmental conditions in which the seasoning is carried out.
- Fermented vegetables: low levels of biogenic amines have been detected in studies related to Asian foods. Not relevant quantities of tyramine have been found in Japanese plants fermented and tyramine and histamine in the Miso, a popular oriental product, obtained from the fermentation of vegetables by yeasts, molds and bacteria (Ibe et al., 1992). Vegetables processed and brined starting from high quality raw materials do not develop high levels of biogenic amines unless they have been contaminated previously or stored at abuse temperature for too long time (Hornero-Mendez and Garrido-Fernandez, 1997). Studies conducted on table olives, frozen spinach puree, ketchup, tomato concentrates and frozen peas showed a limited or no presence of biogenic amines (Kalac et al., 2002).
- Fermented fish products: some studies conducted to determine the content of biogenic amines in this type of products show that the level of such compounds can also be high. Yankah et al. (1993) studied the evolution of nitrogen compounds

during processing and storage of fermented fish products and have detected traces of putrescine, tyramine, agmatine and tryptamine and no histamine. The spermine was the only biogenic amine determined in high quantities after fermentation. Histamine formation, the only biogenic amines for which limits are established, it is very variable as a function of time, temperature and the type and quantity of microorganism's present (Sims et al., 1992).

2.12. COAGULASE-NEGATIVE STAPHYLOCOCCI (CNC)

Spontaneous meat fermentation leads to a variety of different bacteria, among which the LAB are ubiquitous (Ravyts et al., 2010). Within the group of catalase-positive cocci, the species diversity can be considerably larger. *Staphylococcus xylosus* is frequently the most dominant species in European-style traditionally fermented sausages (Cocolin et al., 2011), but *Staphylococcus saprophyticus* and *Staphylococcus equorum* can also dominate the staphylococcal microbiota depending on the type of product (Talon et al., 2007). In addition, a large variety of subdominant fractions can be found, such as S*taphylococcus carnosus, Staphylococcus epidermidis,* S*taphylococcus haemolyticus, Staphylococcus pasteuri, Staphylococcus sciuri, Staphylococcus succinus, Staphylococcus vitulinus,* and *Staphylococcus warneri* (Talon et al., 2007). In industrialized products, both *Staphylococcus carnosus a*nd *Staphylococcus xylosus* are commonly used as starter cultures due to their nitrate-reducing ability and aroma production (Leroy et al., 2006). Besides CNC, *Kocuria* can be part of the catalase-positive coccal microbiota too, among which *Kocuria varians* is the most frequently found representative, often used as a starter culture for similar reasons (Cocconcelli, 2007). Overall, the CNC species diversity of a fermented meat product is difficult to predict, as it is influenced by a combination of complex factors, including the muscle type used, the ingredients added, and the processing conditions applied.

2.12.1. FLAVOUR GENERATION

The metabolic activity of CNC is crucial to flavour development, especially in Mediterranean fermented meats where less acid and more complex sensory profiles are expected (Demeyer et al., 2000). Potential contributions of CNC to flavour development can be divided into four

major modes of action:

(1) carbohydrate fermentation,
(2) amino acid conversion reactions,
(3) lipid β-oxidation,
(4) esterase activities (Flores & Olivares, 2015).

The bacterial fermentation of carbohydrates generates lactic acid, resulting in a pH decrease, the coagulation of meat proteins, and an acid taste. This role is mostly taken up by the LAB (Leroy et al., 2006). CNC also use carbohydrates, albeit slower and in lower quantities, and convert them into organic acids (mostly lactic acid and acetic acid), as well as volatile compounds, such as diacetyl, acetaldehyde, and acetoin. The latter pyruvate-derived compounds can give a buttery note or dairy aroma to dry fermented sausages (Toldrá, 2008).

In addition to carbohydrate metabolism and generally more decisive for flavour, the conversion of amino acids by CNC produces volatile compounds with high aromatic impact (Smit et al., 2009).

Post-mortem, the sarcoplasmic and myofibrillar proteins are enzymatically hydrolyzed by muscle proteinases and peptidases (Toldrá and Reig, 2015). Besides these endogenous enzymes, enzymatic activity by CNC is discernible, in particular on peptides and their conversion into free amino acids (Flores and Toldrá, 2011). This may account for up to 40% of total proteolysis, but becomes inhibited at low pH (Montel et al., 1998). Both peptides and free amino acids can undergo additional reactions to form volatile and non-volatile compounds (Toldrá, 1998). For instance, the CNC group can transaminate and decarboxylate the branched-chain amino acids valine, leucine, and isoleucine into the corresponding branched-chain aldehydes, carboxylic acids, and alcohols (Flores and Olivares, 2015). The compounds originating from leucine, i.e., 3-methyl butanal, 3-methyl butanol, and 3-methyl butanoic acid, have often been associated with fermented sausage aroma (Leroy et al., 2006).

Lipolysis takes place by enzymatic hydrolysis of the lipid fraction present in the muscle and in subcutaneous tissues, consisting mainly of triglycerides (±85%) and phospholipids (±15%). Once more, this is mostly performed by endogenous enzymes, although lipases of CNC can also play a role (Toldrá et al., 2001). The released fatty acids can then be degraded by the activity of CNC enzymes through incomplete β-oxidation. Fatty acids are first

oxidized to enoyl-CoA, which is then hydrated to hydroxyacyl-CoA and further oxidized to ketoacyl-CoA. Ketoacyl-CoA esters are deacylated into β-ketoacids, short-chain free fatty acids, and CoA by thioesterase enzymes (Flores and Olivares, 2015). Decarboxylation reactions of the β-ketoacids by CNC lead to methyl ketones (Demeyer and Stahnke, 2002), such as 2-pentanone, 2-hexanone, and 2-heptanone, which contribute to cured flavour formation (Stahnke, 1999). These methyl ketones can be further converted to secondary alcohols (Montel et al., 1998). Finally, aromatic ester compounds can be generated by esterase activity of CNC through the reaction of an acid and an alcohol (Flores & Olivares, 2015), leading for instance to ethyl esters (Stahnke et al., 2002). Alternatively, these bacterial enzymes are able to hydrolyze ester compounds (Talon et al., 1998).

2.12.2. COLOUR GENERATION

Even though the ability for nitrate reduction is a rather frequent feature within the *Staphylococcus* genus, nitrate reductase activity differs considerably between and within species. Not surprisingly, this technologically important activity is usually strong in the typical starter culture species *Staphylococcus carnosus* and *Staphylococcus xylosus*, notwithstanding strain variability. It also appears to be prominent in other CNC species, such as *S. equorum* and *S. lentus*, and for several strains of *S. simulans*, *S. sciu*ri, and *S.* succinus (Talon et al., 1999). In contrast, nitrate reductase activity is often absent in strains of *S. saprophyticus*, *S. succinus*, and *S. warneri* (Mauriello et al., 2004). A large variability in the degree of conversion was found, whereby only 35 strains showed high conversion under the conditions tested. The latter strains belonged mainly to the species S. *carnosus, S. epidermidis, S. equorum, S. haemolyticus, S. pasteuri,* and *S. xylosus*. For correct colour development with nitrate salts, it is not sufficient to merely rely on the use of a particular CNC strain with a demonstrated nitrate reductase activity. Process conditions should be also considered. It has for instance been established that nitrate reductase activity is maximal during exponential growth and is induced by anaerobic growth conditions in the presence of nitrate (Talon et al., 1999). Also, the meat acidification rate and the final pH are important, since nitrate reductase activity decreases strongly below pH 5.2 (Talon et al., 2004). Moreover, the pH dependency of the emerging CNC background microbiota may be an important parameter to take into account for colour generation when using nitrate salts as

the sole curing agent. For instance, in meat models, mild acidification conditions have been shown to favor nitrate-reducing *S. equorum* strains, while strains belonging to *S. saprophyticus* are favored at faster acidification rates, resulting in poor colour formation (Sánchez Mainar and Leroy, 2015). Although nitrate reduction can be achieved at 15–20 °C, the process seems to be more effective at temperatures above 30 °C (Casaburi et al., 2005).

An innovative but still largely speculative alternative to the generation of fermented meat colour (nitrosomyoglobin) via nitrate reductase activity may be provided by another arginine conversion mechanism. The ability for nitric oxide synthase (NOS) activity leads to the formation of potential colour-yielding NO from arginine (Morita et al., 1998). The NOS system synthesizes NO by oxidation of the guanidium group from arginine, consuming $NADPH^+$, H^+ as a co-factor, and yielding citrulline as a co-product. In humans, NO plays a key role in several physiological and pathological processes, such as cellular signalling and immune response (Moncada et al., 1991). Over the last 15 years, prokaryotic proteins that are homologous to animal NOS have been identified and characterized (Crane et al., 2010). The presence of NOS in bacteria has been well documented for Nocardia, *Salmonella*, and *Bacillus subtilis* (Pant et al., 2002). Theoretically, bacterial NOS (bNOS) from meat-associated bacteria could be used as an alternative NO generator to replace the use of nitrate and/or nitrite. The potential presence of NOS activity in LAB has been discussed in the literature (Yarullina et al., 2006). Yet, these studies need to be considered as preliminary because of insufficient experimental controls, interference due to traces of nitrate present in the cultivation medium, and a lack of a direct measurement of metabolites (Xu et al., 2000). Moreover, none of the genomes of the currently sequenced *Lactobacillus* strains contain a homologue of the nos gene required for NOS activity (Agarwala et al., 2015). For staphylococci, NOS activity has been reported in *S. aureus* (Choi et al., 1997; Hong et al., 2003), but only preliminary suggestions for the presence of NOS in CNS have been made. Morita et al. (1998) have investigated the use of a *S. xylosu*s strain for its ability to convert metmyoglobin into nitrosomyoglobin in the absence of added nitrate and nitrite in a myoglobin-containing cultivation medium at pH 5.8 and 37 °C. In addition, it has been hypothesised that the latter strain is able to contribute to colour formation during the preparation of nitrate- and nitrite-free salami. Gøtterup et al. (2007), however, have not found any evidence of NOS activity in a myoglobin-containing cultivation medium for a

selection of ten CNS strains from cured meat sources, with activity levels possibly being below the detection limit. In *S. aureus*, for instance, NOS activity has been estimated in the magnitude of only nanomole per min per mg of protein (Hong et al., 2003).

2.13. YEASTS

Spontaneous fermentations are generally characterized by the presence of yeasts, but studies on the biodiversity of yeast in sausages are limited. *Debaryomyces hansenii* is the most commonly isolated species of yeast according to various studies, but other kinds of yeast have also been found, such as *Candida* spp. (Gardini et al., 2001). The increase in pH and the decrease in the lactic acid content in sausages can be caused by these yeasts which contribute to the characteristics of the final product (Gardini et al., 2001). Both *Debaryomyces hansenii* and *Candida utilis* initially proliferate in sausages and then slowly decrease (Olesen and Stahnke, 2000). On the contrary, primary and secondary metabolism, where lipases and proteinases are key enzymes, are the main pathways of these organisms to produce energy and can give the typical aroma of products (Cocolin et al., 2006). A yeast can be added as a flavor enhancer and can also stabilize the red color of fermented sausages (Olesen and Stahnke, 2000).

Debaryomyces spp. are extremophile yeasts, perfect, haploid that reproduce asexually from multilateral spores, pseudomycelius is absent, primitive or occasionally well developed. Heterogamous conjugation is the way to sexual reproduction. The development of the typical sausage aroma was possible through the inhibition of the generation of lipid oxidation products and by promoting the generation of ethyl ester.

Cocolin et al. (2006) used a multi-phase approach during the fermentation of a traditional sausage produced in Northern Italy. Culture-dependent and independent methods were used to profile the yeast communities present during maturation. Through molecular identification by PCR-DGGE and sequencing of the partial 26S rRNA encoding gene of 180 isolates, *Debaryomyces hansenii* was found to be the dominant species during the fermentation process.

Although the origin of meat and the factory environment have been reported as factors that can cause variations in yeast populations in fermented meat products, most studies indicate *Debaryomyces hansenii* as the most frequently and abundantly isolated (Flores et al., 2015).

2.14. MOLDS

The surface of seasoned meat is colonized by molds capable of growing on different environments and substrates (Magistà et al., 2017).

The xerotolerant and xerophilic fungi preferably grow in an environment with low water activity and high salt concentrations such as salami. In this type of product, fungi also play an important role in the production process because they can lead to the development of specific aromas and flavor, due to their lipolytic and proteolytic activities (Sonjak et al., 2011).

The genus *Penicillium* represents the main population of molds of the surface microbiota on products based on dry meat (Sonjak et al., 2011). The *Penicillium* is one of the most common fungi that can grow in a wide range of habitats, from soil to vegetation, air, to the interior and to the various foodstuffs (Visagie et al., 2014).

Important taxonomic characters of *Penicillium* are the presence of conidiophore (when produced). The branching patterns of the conidiophore have traditionally been used in the classification of *Penicillium* (Visagie et al., 2014).

Penicillium species have been found in fermented meat sausages and are responsible for superficial colonization, especially *Penicillum nalgiovense* and, to a lesser extent, *Penicillum chrysogenum* (López- Dıáz et al., 2001).

This layer of mold is important for the sausage because it has an antioxidant effect, protecting from the development of rancidity and maintaining the color, and gives to the sausage its typical aspect because it allows the development of a positive microclimate on the surface to prevent, for example, the formation of crust or too viscosity of the surface (Visagie et al., 2014).

CHAPTER 3

LACTOBACILLUS SAKEI

3.1. *LACTOBACILLUS SAKEI*

Described for the first time almost a century ago as a contaminant of the "Saké" rice wine (Katagiri et al ., 1934), a product from which it later took its name, *Lactobacillus sakei* is able to colonize very different habitats (Zagorec and Champomier-Vergès , 2017). It was isolated for the first time in products of animal and vegetable origin (Langella et al., 1996). This microorganism is used in many fermented products both to produce molecules that contribute to the organoleptic characteristics, and for the formation of substances capable of inhibiting the growth of degradative microorganisms but, above all, pathogenic microorganism *Staphylococcus.* *L. sakei* is considered as the LAB characteristic of meat products, both raw, preserved at low temperature and vacuum packed, and fermented product. Being therefore present in many food products, it is considered as a microorganism belonging to the human diet, in fact it has also been also isolated from human faeces, although the gastrointestinal tract is not its optimal environment (Chiaramente et al., 2009). Among LAB isolated in salami, *Lactobacillus sakei*, *Lactobacillus plantarum* and *Lactobacillus curvatus* are those most present, but *L. sakei* is the predominant specie. For this reason, it is commonly used as a starter culture (Leroy et al., 2006) for several factors: it is able to obtain energy from amino acids, if the concentration of sugars is low, and determines the formation of particular aromatic compounds that allow to characterize the final product. *L. sakei* appears to be a very resistant microorganism able to grow at refrigeration temperatures and in the presence of even high salt concentration. In fact, it is a mesophilic microorganism with an optimal temperature of 30-35 ° C, but capable of resisting even at temperatures that drop to 15 ° C as previously mentioned. There are also *L. sakei* strains able to withstand salt concentrations up to 10%, a very important feature because it allows its use as starter cultures to guide the fermentation process of various meat. This peculiarity immediately made them the main LAB responsible for spontaneous fermentation, and consequently, in this matrix it carries out its main role of acidifying the salamis themselves (McLeod et al., 2008), as previously mentioned. It is part of the Gram-positive group and belongs to group II of *Lactobacillus* (Figure 3.1.).

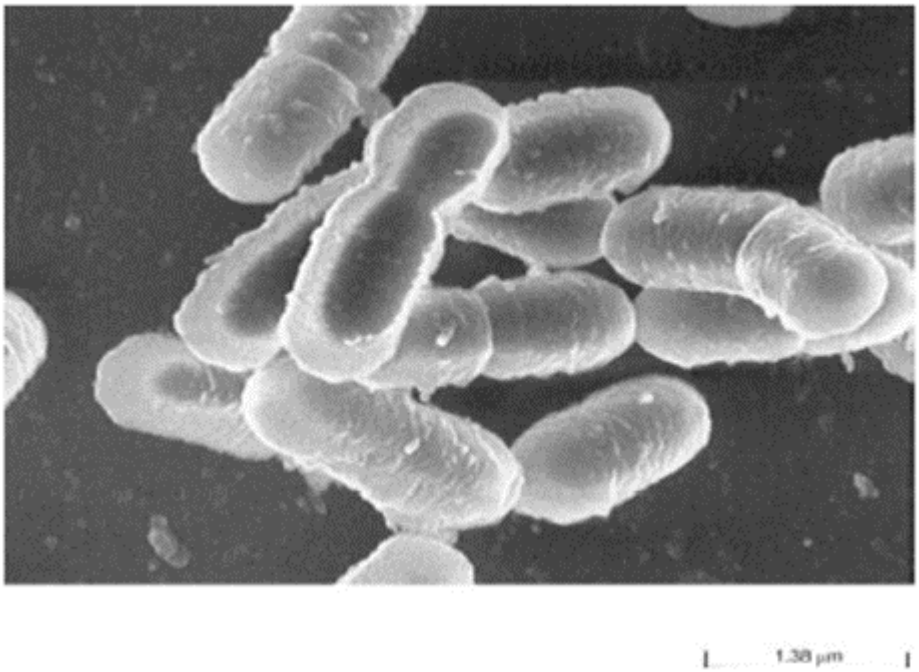

Figure 3.1.: Scanning electron microscopy of *L. sakei* strain 23K grown at 30 ° C

As regards the genetic heritage of *L. sakei*, it appears to be broad and with phenotypic diversity and through recent studies on the genome sequence a great variability in size has been observed. The average size was 2,020 kb with a variation of about 25% (from 1,814 to 2,309 kb) (Chaillou et al., 2009). Further analyzes on the genome have highlighted some specific traits of this species, which explain the high adaptation in the meat environment (Claesson et al., 2007).

For his activity as a starter culture, *L. sakei* represents the predominant component in the first phase of fermentation, in which all the fermentable carbohydrates are consumed in a few days or sometimes in a few hours. After this initial phase, *L. sakei* modifies its metabolism to carry out activities defined as "secondary" and which influence the consistency, the aromatic profile of foods and their nutritional value.

The aromas present in these fermented products are obtained from the production of volatile and aromatic compounds deriving from different chemical classes such as alcohols, aldehydes, fatty acids, esters, sulfur compounds and ketons. The origin of these compounds is due to the biochemical transformation of carbohydrates, proteins and lipid.

The main metabolic activities of *L. sakei* are related to the amino acids, both branched and sulfurized, and to the metabolism of products derived from carbohydrates. Through these

metabolisms, energy is produced in the form of ATP and this allows the survival of *L. sakei* also in environments characterized by adverse nutritional and environmental conditions that would be lethal. There are many conversions of branched amino acids that can be attributed to this microorganism, but it has not yet been determined how the cell addresses and chooses between the various available routes and how many of them then contribute to the final energy balance.

The different metabolic pathways present in *L. sakei* can be used to research and obtain diversified fermented products through peculiar aromatic substances different from the traditional ones, or that anyway allow to be considered innovative. Through the modification of chemico-physical parameters such as fermentation temperature, pH, salt concentration, osmolarity or the composition of other nutrients, metabolic performances can be influenced (Smid and Kleerebezem, 2014).

3.2. CARBOHYDRATE METABOLISM

In meat the main sugars are glucose, derived from glycogen, and ribose which is formed by hydrolysis of ATP. Since the meat is a matrix poor in fermentable sugars, these are usually added to the mixture in the form of glucose, sucrose and lactose depending on the speed of acidification to be obtained, in order to speed up the fermentation process and the subsequent maturation of the meat product.

As regards the fermentation of hexose sugars by *L. sakei*, it turns out to be homolactic and occurs via glycolysis with production of lactic acid, a component that determines the lowering of the pH. Glucose can be transported into the cell via the PTS system (carbohydrate transferase) or via non-PTS secondary pathways, while the transport of lactose and galactose does not depend on a PTS complex (Lauret et al., 1996). The β-galactosidase protein from *L. sakei* is encoded in the chromosome by the two lacL and lacM genes which, overlapping by 15 nucleotides, appear to be outside the cellular structure so the regulation is coordinated to produce the same quantity of both polypeptides (Obst et al., 1995).

The transport of lactose remains unknown, but it enters the cell as lactose-phosphate, which is a phosphorylated molecule, which will then be split into glucose so that it can proceed through the glycolytic pathway (Champomier-Vergès et al., 2001). As for

ribose, arabinose and gluconate, fermentation appears to be heterofermentative via the phosphoketolase pathway but thiamine (vitamin B), or one of its precursors, is required in the growth medium to be used (Stentz e Zagorec, 1999).

During the fermentation of sugars, both D- and L-lactate are produced, however in *L. sakei* is present only L-lactate dehydrogenase, encoded by the monocistronic gene ldHL whose interruption is sufficient to prevent the production of both D- and L-lactate. Conversion from L- to D-lactate is catalyzed by lactate racemase, not yet characterized at the genetic level (Hiyama et al., 1968).

As indicated in Figure 3.2., in conditions of glucose deficiency also arginine can be catabolized, by means of the enzyme arginine-deaminase, through a special way used to produce alternative energy.

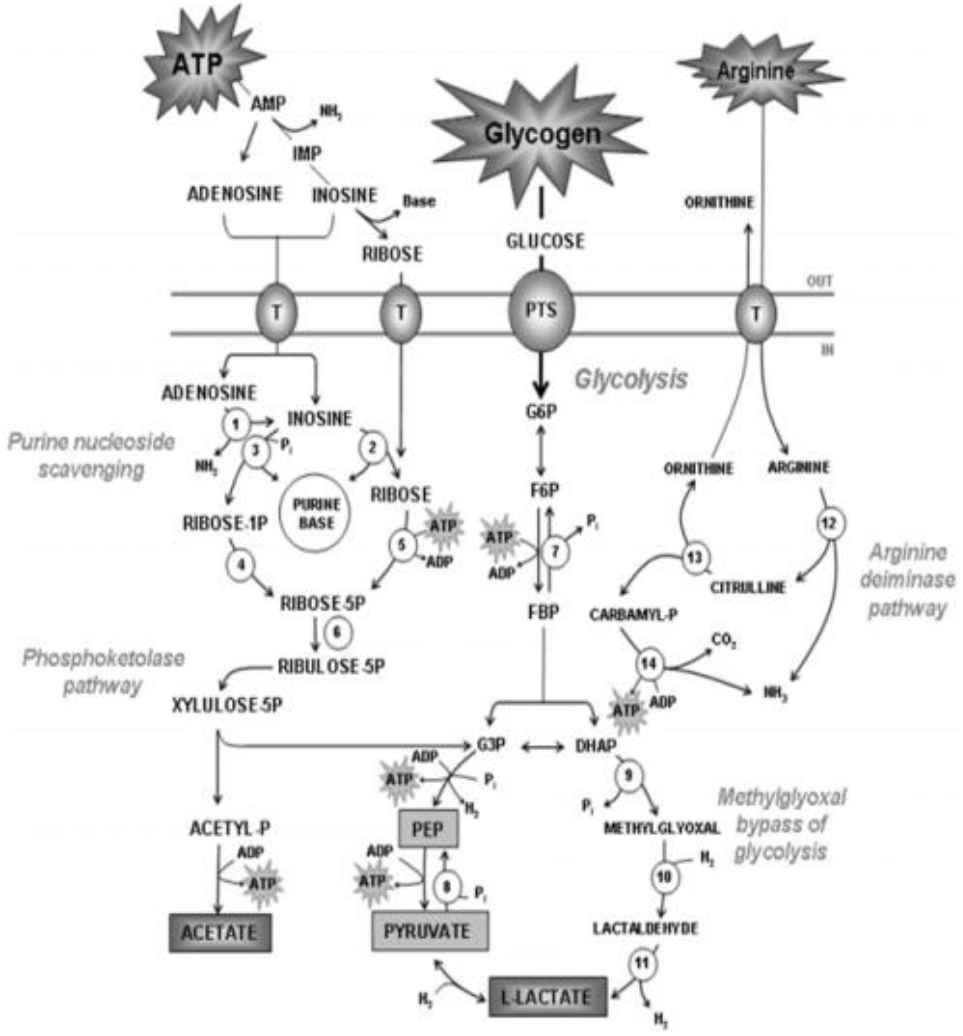

Figure 3.2.: Overview of the fermentation path of *L. sakei* following its adaptation in meat (Chaillou et al., 2005).

3.3. AMINO ACID CATABOLISM

The meat is a substrate composed of numerous proteins and during its aging the proteolytic enzymes are released by lysosomes, so the amino acids are released from the meat proteins even without bacterial activity. Amino acids play a key role in the survival and growth of *L. sakei*, which appears to be an auxotrophic microorganism for all amino acids, with the exception of aspartic acid and glutamic acid obtained from the deamination of asparagine and glutamine, respectively (Chaillou et al., 2005). The absence of a metabolic pathway for amino acid synthesis and the absence of transaminases are the result of the adaptation of *L. sakei* in meat (Sinz and Schwab, 2012). The amino acids used contribute both to the generation of energy and to the production of aroma components that characterize the final product but can also lead to the formation of biogenic amines (Smid and Kleerebezem, 2014). As will be discussed further below, *L. sakei* is able to degrade arginine, which is important for its survival, and furthermore this action is essential for the differentiation from *L. curvatus.* Other amino acids, such as serine (Figure 3.3.), asparagine, cysteine and methionine, are metabolized through potentially energetic pathways that start from pyruvate accumulation (Montanari et al., 2018). The conversion of serine to pyruvate has been described in *Pediococcus pentosaceus*, as the result of the activity of the enzyme serine dehydrate and without the intervention of transaminases, which are absent in *L. sakei* (Irmler et al., 2013). Through the degradation of this amino acid there is the consequent formation of formate, succinate and acetate, as described also for *L. plantarum* (Skeie et al., 2008). McLeod et al. (2017) observed in *L. sakei* a drastic use of serine and asparagine in conjunction with growth in an environment with a low glucose concentration, but an increase in the production of L-serine dehydratase was also observed.

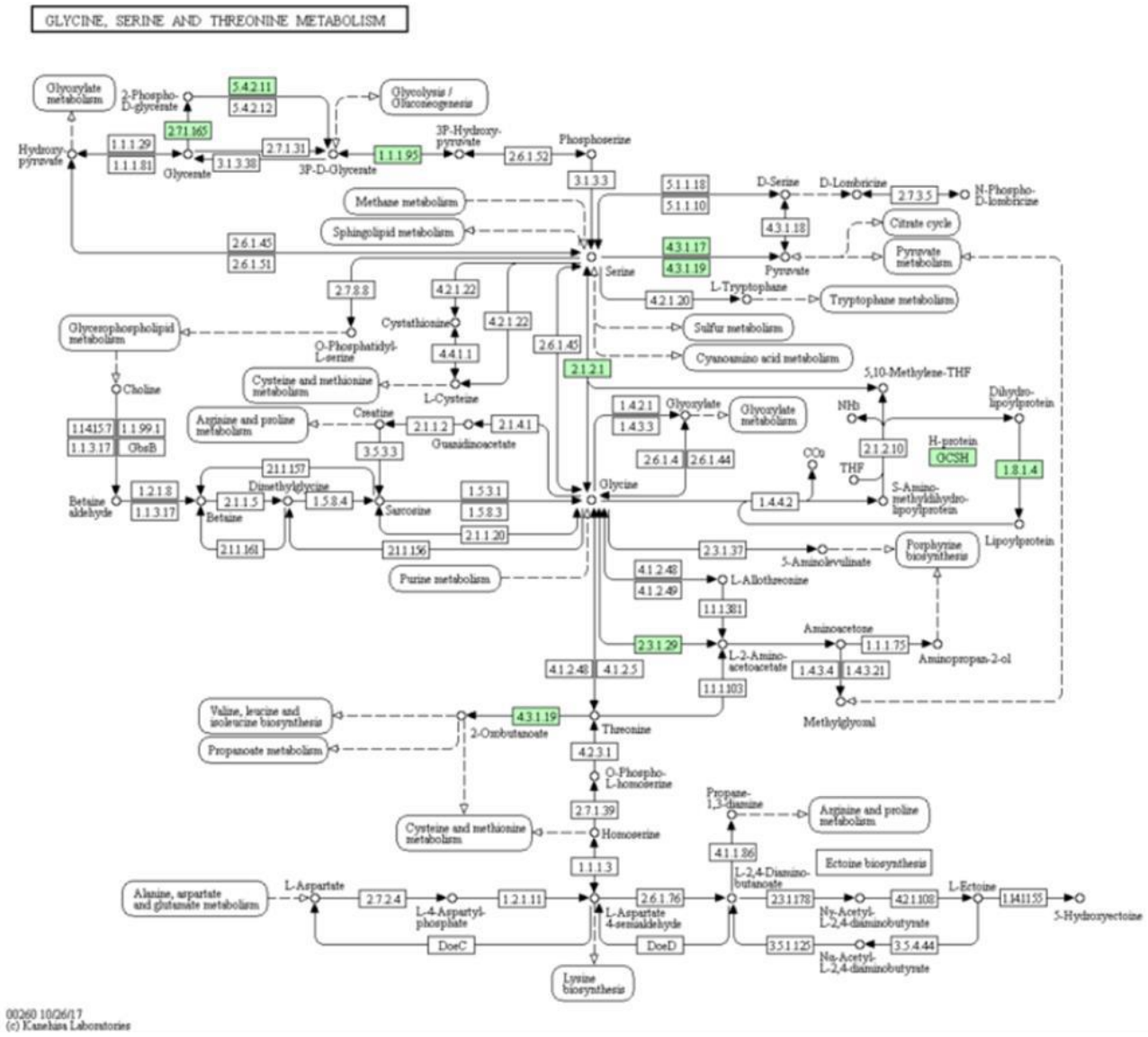

Figure 3.3.: Glycine, serine and threonine metabolism of *L. sakei* (KEGG database). Serine derives from 3-phospho-D-glycerate, an intermediate of glycolysis, while glycine derives from serine. Threonine is an essential amino acid that animals cannot synthesize, whereas in bacteria and plants it derives from aspartate

Asparagine can be converted to aspartic acid with release of ammonia following the action of asparaginase. Furthermore, cysteine can be metabolized into pyruvate, ammonia and hydrogen sulfide (Figure 3.4.) (Fernández and Zúñiga, 2006). The enzyme responsible for this pathway is cystathionine-y-lyase, found in *Lactobacillus fermentum* (Smacchi e Gobbetti, 1998), *Lactobacillus lactis* (Fernández et al., 2002) and *Lactobacillus reuteri* (Lo et al., 2009).

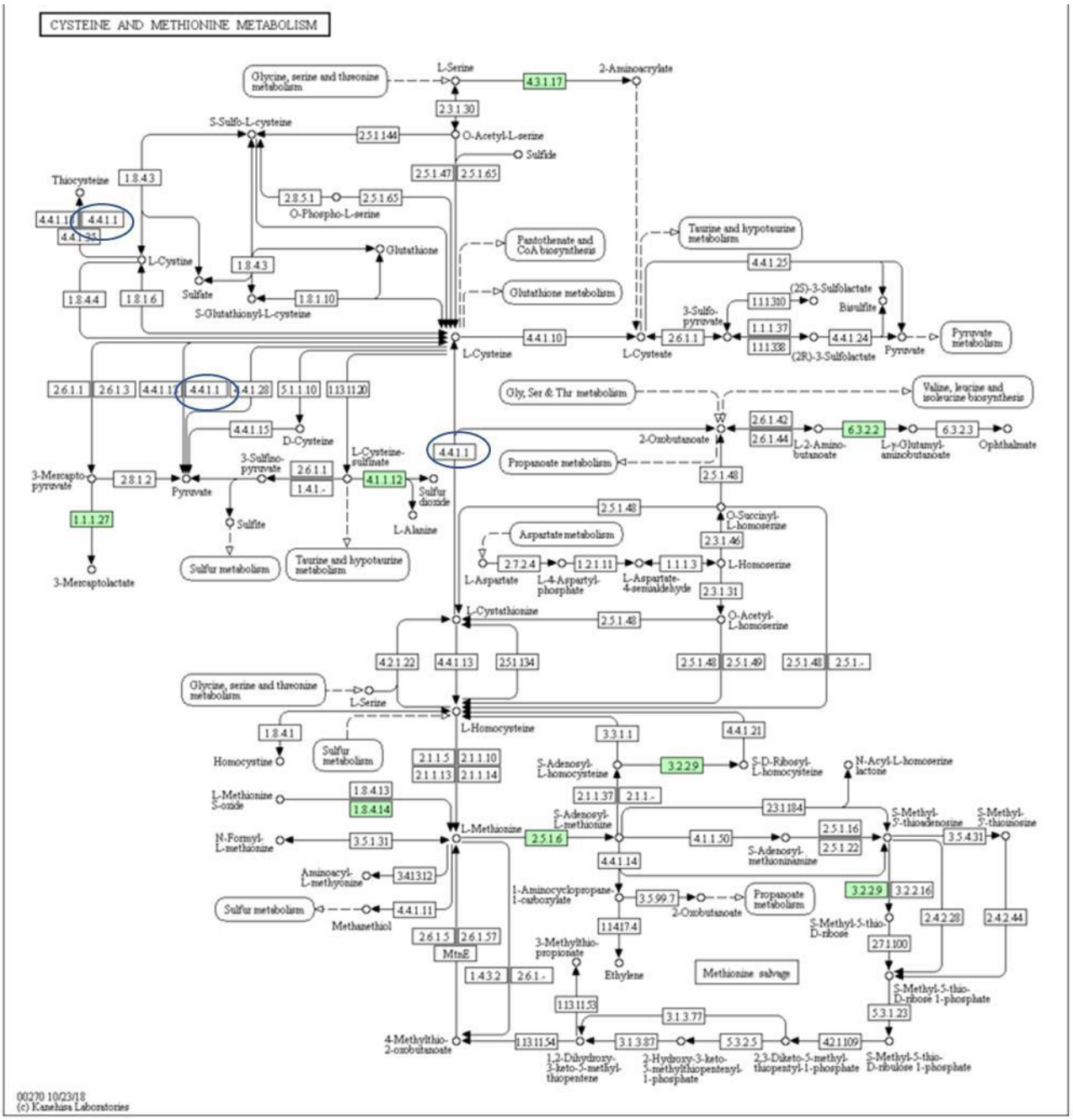

Figure 3.4.: Cysteine and methionine metabolism in *L. sakei* (KEGG database). Cysteine and methionine are sulfur-containing amino acids. Cysteine is synthesized by serine through the enzyme acetylserine by transferring hydrogen sulfide, and then metabolized by pyruvate. Methionine is an essential amino acid, synthesized from aspartate in bacteria and plants.

3.4. RESPONSE TO STRESS

Numerous factors associated with technological processes correspond to "stress" factors for the growth of microorganisms. These factors correspond to the presence of high salt concentrations, presence of nitrite/nitrate, refrigeration, production of an artificially modified atmosphere in order to reduce the redox potential and oxygen toxicity.

L. sakei is well equipped to withstand these conditions, in fact it has the ability to accumulate osmo and cryoprotective solutes, such as betaine and carnitine, which play an important role in the acclimation of *L. sakei*. The accumulation of these compounds appears to be guided by three absorption systems: LSA0616 to LSA0619, from LSA1694 to LSA1696, LSA1869-LSA1870 and they are coupled to a mechanism of sympathetic Na^+ dependent (LSA0264) (Chaillou et al., 2005). Furthermore, some genes responsible for the response to heat stress (*hrc*A, *gro*E, *dna*K and *dna*J) have been identified, which code for some proteins that are highly conserved in all microorganisms (Schmidt et al., 1999). The transcription of these genes is induced both in conditions of thermal shock and in the presence of salt and ethyl alcohol.

Since the surface of the flesh is exposed to oxygen, the ability to deal with oxidative stress is important in microorganisms to be competitive. *L. sakei* has mechanisms of response to oxidative stress induced by oxygen radicals and H_2O_2, in fact it has a heme-dependent catalase (katA) responsible for an efficient decomposition of H_2O_2. The expression of this enzyme is regulated by the presence of the radicals or by the passage from an anaerobic condition to the aerobic one (Hertel et al., 1998).

3.5. PRODUCTION OF EXOPOLYSACCHARIDES

Some strains of *L. sakei* can produce exopolysaccharides (EPS), or extracellular polymers consisting of 2 to 8 sub-units, of variable length and which can be soluble in water. The production of these substances does not always represent a desired property in the final product, in fact they can cause important deterioration in cooked meat if vacuum packed (Champomier-Vergèrs et al., 2001). The exopolysaccharides produced by *L. sakei* consist of glucose and rhamnose in a ratio of 3:2 (Van den Berg et al., 1995), or from glucose and galactose (Kunene et al., 2000).

It was shown that the composition of the EPS produced is not altered when the cells are grown in different energy sources, but the production yield is greater when glucose is used (Van den Berg et al., 1995).

3.6. BACTERIOCINS

Bacteriocins are peptides or proteins with antimicrobial activity against other Gram-positive bacteria, including food-degrading bacteria and pathogenic strains (De Vuyst and Vandamme, 1994). Most of them are small, heat resistant and have a high isoelectric point.

The bacteriocins most produced by *L. sakei* are the sakacine, in particular the most frequently isolated are: Sakacina A, Sakacina P and Lactocina S (Nettles and Barefort, 1993). Furthermore, another bacteriocin produced by the *L. sakei* strain 2512 was isolated and purified, and named Sakacina G (Simon et al., 2002).

The production of Sakacin A and Lactocin S is associated with two plasmids, respectively, one of 28 Kb and the other of 50Kb (Champomier-Vergès et al., 2001). The Sakacina P is the bacteriocin able to inhibit the growth of *Listeria monocytogenes* and to determine a decrease in the growth of enterococci (Urso et al., 2006b). Some studies have also shown that the inhibition of growth of *L. monocytogenes* was determined precisely by Sakacina P as inoculated directly into the finished product, thus being able to exclude the inhibitory action (Figure 3.5.) (Zdolec et al., 2007).

Bacteriocin	Producers	Biochemical characteristics	Inhibitory spectrum
sakacin A	*L. sakei* L706	class IIa bacteriocin 4309 Da 41 amino acids hydrophobic identical to curvacin A produced by *L. curvatus* LTH1174	*Carnobacterium piscicola* *Enterococcus* spp. *L. sakei, L. curvatus,* *Lactobacillus brevis* *Leuconostoc paramesenteroides* *Listeria monocytogenes* *Staphylococcus aureus* *Lactococcus cremoris*
sakacin P (also called sakacin 674)	*L. sakei* LTH673 *L. sakei* L674	class IIa bacteriocin 4436 Da 43 amino acids hydrophobic	*L. curvatus, Lactobacillus delbrueckii, L. sakei* *L. pentosus, L. plantarum, Lactobacillus reuteri* *L. fructivorans , Listeria ivanovii,* *E. faecalis,* *Carnobacterium* spp. *Listeria monocytogenes*
lactocin S (also called sakacin M)	*L. sakei* L45 *L. sakei* 148	antibiotic 3769 Da 33 amino acids	*Lactobacillus* spp. *Leuconostoc* spp. *Pediococcus* spp.

Figure 3.5.: Biochemical characteristics and inhibitory spectrum of bacteriocins produced by *L. sakei* (Champomier-Vergès et al., 2001)

3.7. ARGININE METABOLISM

As seen previously, *L. sakei* is able to use arginine to obtain energy only in the presence of low concentrations of glucose and this phenomenon is associated with a greater survival of the microorganism even for long periods (Champomier-Vergès et al., 1999).

The alternative metabolic pathway ADI (arginine deaminase), schematized in Figure 3.6., is composed of three enzymes: arginine deaminase, ornithine transferase and carbamato-kinase (CK) in association with the arginine/ornithine antiport. Through this metabolic pathway, as can be seen from Figure 3.6., the amino acid is degraded with the formation of ammonia (NH_3), 1 mole of ATP per mole of consumed arginine, ornithine and carbon dioxide. In this way, *L. sakei* gains a considerable competitive advantage: in fact, the ATP generated therein provides additional energy, while the formation of ammonia offers an advantage in conditions of stress with acidic pH. The genes involved in arginine degradation are: *arc*A (ADI), *arb*B (cOTD), *arc*C (CK) and *arc*D (anti-port) and usually form an operon, whose organization and transcription may vary in different species (Champomier Vergès et al., 1999). The expression of this pathway in *L. sakei* is stimulated by anaerobiosis and a study by Rimaux et al. (2012) showed that the pH influences the conversion of arginine.

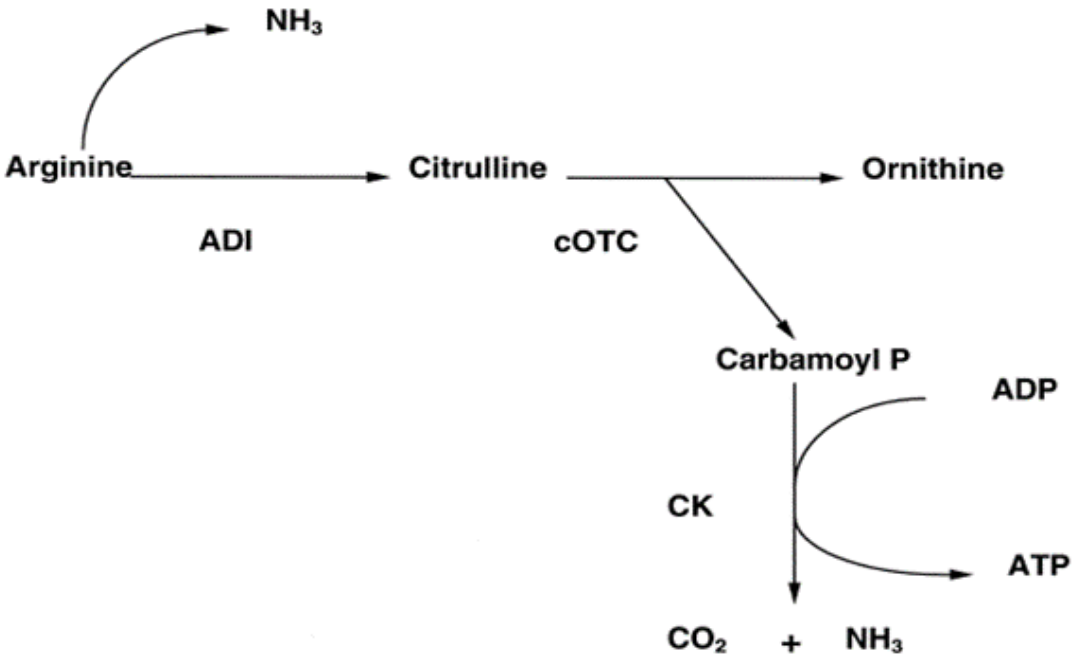

Figure 3.6.: Schematic representation of the arginine deaminase pathway (ADI) (Champomier Vergès et al., 1999)

3.8. PROTEOLYTIC ACTIVITY

As already mentioned, in fermented meat products LAB, such as *Lactobacillus sakei,* play a fundamental role in the fermentation process, the protection of food from the proliferation of pathogenic microorganisms and the formation of aromatic compound. Recent studies have shown that the species of *Lactobacillus* most commonly found in fermented meats, for example *Lactobacillus sakei, Lactobacillus curvatus* and *Lactobacillus plantarum,* are able to hydrolyze myofibrillary and sarcoplasmic muscle proteins in vitro (Sanz and Toldrii, 2001).

The hydrophilic peptides, deriving from the hydrophilic activity of some species on the water-soluble proteins, play an important role in the determination of the final aroma (Fadda et al., 1999). Some peptidases have been isolated from lactobacilli used in meat fermentation and only three peptidases have been purified and studied in *L. sakei*: an aminopeptidase with broad specificity, a dipeptidase with primary specificity with respect to Ala-X peptides and neutral amino acids, an X- propyl - dipeptil- aminotransferase specific for X-Pro peptides and a tripeptidase with wide specificity towards di - and tripeptides (Champomier-Vergès et al., 2001).

AIM

Lactobacillus sakei is a lactic acid bacteria (LAB) highly adapted to meat environments, in which it can rapidly grow and compete with other species occurring in these matrices, including pathogens or spoilage microflora (Chaillou et al., 2013). This adaption to an environmental niche explains specific features of this species, such as the absence of transaminase, the auxotrophy for all the amino acids, apart from aspartate and glutamate (Chaillou et al., 2005), and the ability to produce energy from alternative substrates when hexose sugars are completely depleted. Indeed, *L. sakei* can ferment also pentoses contained in the nucleosides via phospoketolase pathway (McLeod et al., 2008) and can gain energy also from the catabolism of amino acids, mainly arginine through arginine deiminase pathway (Rimaux et al., 2012) or serine, that can be deaminated by L-serine dehydratase to obtain a surplus of pyruvate (Irmler et al., 2013).

These metabolic peculiarities make this species dominant in meat products, in which is also often responsible for the natural fermentation of dry fermented sausages (Hugas et al., 1993). Moreover, thanks to the capacity to produce bacteriocins able to inhibit pathogenic bacteria, as well as to the high adaptability to strict environment conditions such as low temperature and high salt concentration (Belfiore et al., 2013; Duhutrel et al., 2010), selected strains of *L. sakei* species are widely used as starter cultures in meat fermentation, together with other LAB such as *Lactobacillus curvatus*, *Pediococcus pentosaceus* and *P. acidilactici* (Champomier-Vergès et al., 2001; Hammes and Hertel, 1998).

Starting from these considerations, the general aim of this thesis was to investigate the metabolic and physiological reasons that underlie these specific features of *L. sakei*, in particular its ability to well survive in fermented meats for long period.

From a technological point of view, the acquired knowledge can improve and optimize the use of this starter culture in food industry.

Indeed, it is known that the improper use of starter cultures can result in the proliferation of spoilage microflora or pathogens, that affect the safety and quality of the final product. Moreover, the addition of selected cultures with specific metabolic traits can help to enrich and differentiate the aromatic profiles of fermented sausages, while guaranteeing their quality.

To this purpose, six *L. sakei* strains, isolated from different origins, were first screened to assess growth performances (in relation to temperature and salt concentration) and their

metabolic pathways in terms of amino acid consumption and metabolite (organic acids, C4 volatile compounds) accumulation in a chemically defined medium.

Then, the strain *L. sakei* Chr82 was selected for further studies, in which its adaption and growth ability, as well as its metabolic activity, were investigated in relation to the type (glucose or ribose) and concentration of sugars added to the medium. In this part of the research different analytical approaches were used:

- Traditional microbiological methods (plate counting, OD measure)
- HPLC analysis for amino acid consumption and organic acid accumulation
- ^{1}H-NMR for metabolome analysis
- Flow cytometry to monitor the physiological state (i.e. viability) of *L. sakei* cells.

EXPERIMENTAL PART

CHAPTER 4

IMPROPER USE OF STARTER CULTURES IN FERMENTED MEATS: A CASE STUDY REGARDING A TYPICAL ITALIAN SALAMI (VENTRICINA)

4.1. INTRODUCTION

Ventricina is a fermented sausage typically produced in some areas comprised between Abruzzo and Molise Regions. In spite of the wide variability of local recipes, Ventricina is usually obtained by cutting pork lean meat, which represent about 80% of sausage, in cubes rather big (3-4 cm) while the remaining fat part is constituted by bacon and thigh fat. In addition to salt and pepper, sometimes powdered sweet pepperoni and fennel flower/seed may be added. Traditionally this sausage was stuffed in the stomach of the pork (explaining the etymology of the Italian name: *ventre* = stomach) for ripening. Nowadays it is stuffed in natural (pig bladder or veal caecum) or synthetic casings and ripened at low temperature for at least 100 days. The weight of the final product ranges from 1 to 2.5 kg. After the first 50 days of ripening the external part is covered with pork lard to avoid excessive water losses (Tremonte et al., 2017; Tremonte et al., 2005).

In the absence of specific constraints due to protective marks (PDO, PGI), Ventricina may be produced everywhere and many fermented meat industries, located outside the traditional Regions of Italy, produce this kind of sausage.

Likewise many other traditional salamis, the traditional practices for the production of Ventricina have been modified applying industrial processes able to guarantee the safety and the standardization of the product, especially in the framework of the drastic reduction of salt following the request of markets, nutritionists and consumers. This approach firstly requires the addition of starter cultures, of sugars and a rigorous control of temperature and humidity during fermentation and ripening (Leroy et al., 2015).

For these reasons, the use of starter cultures (lactic acid bacteria, staphylococci and moulds) became in the last decades a widespread operation in the industry. Nevertheless, this practice is still applied in many cases as an empirical operation without the needed attention due to this crucial step for sausage production. A not adequate activity of starter cultures may determine several problems in the fermented sausages, raising both organoleptic and safety concerns. Differently from baking and dairy industries, the use of selected microorganisms is sometime perceived by industrial operators as the adjunct of an additive and not as the adjunct of a viable substrate which has to carry out an essential role in the productive process. This role is dependent on many process factors and raw material

characteristics, first of all the microbial quality of the meat used, i.e. the qualitative and quantitative presence of wild microorganisms.

The starter cultures for meat fermentation are usually composed by lactic acid bacteria (lactobacilli and pediococci) and staphylococci whose growth in the meat mixture is essential to inhibit the proliferation of pathogens, to impart the desired flavor, to control the colour formation and to favor a correct water loss. For many fermented sausages produced in the Mediterranean area, also selected moulds are used for a proper ripening of fermented sausages. Nevertheless, several small and medium industries still prefer the wild "home" fungal microflora for the colonization of casing surface, with several risks concerning the uniformity of colonization, as well as the possible production of mycotoxins.

This work is aimed to describe the defects in an industrial production of Ventricina as the results of the competition between the starter culture added during manufacturing and the wild microrganisms present in the raw materials, which impede a correct colonization of sausages by the selected strains. Two industrial lots were produced to assess the effect of the replacement of the wild fungal microflora with a selected culture of *Penicillium nalgiovense*. The sausages ripened with the wild microflora showed organoleptic defects during the ripening, such as presence of off-odours and crust formation. Also, the sample added with *P. nalgiovense* shower some defects at a lesser extent. For this reason, the evolution of the microbial communities was studied during the different steps of production and ripening and at the end of ripening a metagenomic analysis was performed on the two products taking into consideration two different sections of the sausages, i.e. the internal part and the external part. In addition, analyses concerning the aroma profile characterization and the biogenic amine concentration were carried out.

4.2. MATERIALS AND METHODS

4.2.1. SAUSAGES MANUFACTURE

The samples of Ventricina used in this study were industrially produced in a company located in North Italy using frozen pork shoulder (90%) and lard cubes. The meat was minced (10 mm) and added with dextrose (0.1%), sodium chloride (2.6%), spices (black pepper, red pepper, fennel), nitrates and nitrites, starter cultures (*Lactobacillus sakei*, *Pediococcus pentosaceus*, *Staphylococcus xylosus* and *Staphylococcus carnosus*, all provided by Sacco, Italy). The meat mixtures were stuffed in a synthetic collagen casing

(diameter 11 cm) and eventually immersed in a spore suspension of *Penicillium nalgiovense*. Samples were analysed during fermentation (after 2 days from casing), at the end of drying (7 days from casing) and during ripening (13, 28, 48, 69 and 100 days from casing).

4.2.2. WEIGHT LOSS, A_W AND PH

Sample were weighed during the production and ripening period to calculate the mean weight loss (%) with respect to the initial one; a_w was measured in triplicate with an Aqualab CX3-TE (Labo-Scientifica, Parma, Italy). The pH of fermented sausages during fermentation and ripening was determined in the inner and outer part of the product using a pH-meter Basic 20 (Crison Instruments, Barcelona, Spain).

4.2.3. MICROBIAL COUNTS

After aseptically removing the casing, approx. 10 g of sausage were 10-fold diluted with 0.9% (w/v) NaCl and homogenized in a Lab Blender Stomacher (Seward Medical, London, UK) for 2 min. Decimal dilutions were performed and plated onto selective media. Counts of lactic acid bacteria (LAB) were carried out by plating appropriate dilutions on MRS agar incubated at 30 °C for 48 h in anaerobic conditions. Staphylococci and enterococci were counted by surface-plating on Baird-Parker (added with egg yolk tellurite emulsion), and Slanetz and Bartley medium incubated at 30°C for 48 h and 44°C for 24 h, respectively. *Enterobacteriaceae* were enumerated by pour plating in Violet Red Bile Glucose agar at 37°C for 24 h, while coliform counts were made on Violet Red Bile Agar incubated for 24 h at 37°C (total coliforms) or 44°C (fecal coliforms). Total mesophilic count was determined on Plate Count Agar (28°C for 48 h), while Pseudomonadaceae counts were performed on Pseudomonas agar base, supplemented with Pseudomonas CFC Supplement and incubated at 30 °C for 48 h. All the media were purchased from Oxoid, Basingstoke (UK).

4.2.4. DNA EXTRACTION AND SEQUENCING

Total genomic DNA was directly extracted from 10 g of frozen Ventricina. The samples were dissolved in 90 ml of physiological solution (0.9% NaCl) and homogenized in stomacher for 4 minutes at 430 beats per minute. After decanting, 1 ml of the supernatant was collected and subjected to enzymatic treatment towards bacteria (lysozyme) and yeasts

(lyticase) at 37 °C for 1 hour, followed by alkaline lysis with the addition of NaOH and SDS at a final concentration of 0.1 N and 1%, respectively. The extracted DNA was purified by chloroform:isoamyl alcohol 24:1 treatment and precipitated in 0.54 volumes of isopropanol. Finally, the purified DNA was resuspended in water and quantified using Qubit 4 Fluorimeter (ThermoFisher Scientific, Waltham, MA USA). The concentration of the DNA samples was normalized, and the sequencing was carried out through Illumina MiSeq platform which generated 300 bp pair-end sequencing reads. The library for Illumina sequencing was generated from V3-V4 variable regions of ribosomal 16S rRNA in order to characterize the bacterial population of the samples.

4.2.5. BIOINFORMATIC ANALYSIS

FASTQ sequence files from Illumina reads were generated using bcl2fastq2 version 2.18. Initial quality assessment was based on data passing the Illumina Chastity filtering. Subsequently, reads containing PhiX control signal were removed using an in-house filtering protocol. In addition, reads containing (partial) adapters were clipped (up to a minimum read length of 50 bp). A final quality assessment was performed on the remaining reads using the FASTQC quality control tool version 0.11.5. The FASTQ sequences obtained were analyzed using DADA2 versione 1.8 by R 3.5.1 environment. DADA2 implements a new quality-aware model of Illumina amplicon errors without constructing OTUs (Callahan et al., 2016). DADA2 was run as described in https://benjjneb.github.io/dada2/tutorial.html applying the following parameters: trimLeft equal to 30 and truncLen option set to 270 and 200 for the forward and reverse fastq files, respectively. The taxonomic assignment was performed comparing the amplicon sequence variant (ASV) predicted from DADA2 against SILVA database (version 128, https://www.arb-silva.de/documentation/release-128/). ASVs belonging to taxa classified as external sample (Davis et al., 2018) contaminations were not included in the composition analysis for microbial population.

4.2.6. ORGANIC ACID QUANTIFICATION

The extraction of organic acid was performed according to Montanari et al. (2018a): 10 g of each sample were added with 40 ml of 0.05 mM sulfuric solution, homogenized for 10 min

by an Omni Mixer Homogenizer (Omni International, Warrengton, VA, USA) and filtered through a 0.22 μm filter. The extracts analysis was performed by using an HPLC (PU-2089 Intelligent HPLC quaternary pump, UV–VIS multi-wavelength detector UV 2070 Plus, Jasco Corp., Tokio, Japan) and a manual Rheodyne injector with a 20 μl loop (Rheodyne, Rohnert Park, Calif., USA), equipped with a Bio-Rad Aminex (Bio-Rad Laboratories, Hertfordshire, UK) HPX-87H column (300×7.8 mm). The following conditions were used: mobile phase, 0.005 M sulfuric acid; flow rate, 0.60 ml/min; temperature, 65 °C; the UV detector was set at 210 nm. Chromatographic peaks were identified by comparing retention times with those of standards (Sigma-Aldrich, St. Louis, MO) and quantification was carried out by using the external standard method.

4.2.7. BIOGENIC AMINE CONTENT

For the detection of biogenic amines content, samples were subjected to an extraction with trichloroacetic acid followed by a derivatization with dansyl chloride, as described by Pasini et al. (2018). The biogenic amines content was analyzed using a HPLC Agilent Technologies 1260 Infinity with a UV detector (G1314F VWD 1260) at 254 nm. The amounts of amines were expressed as mg/L by reference to a calibration curve obtained with aqueous biogenic amine standards derivatized as described for the samples.

4.2.8. AROMA PROFILE ANALYSIS

Volatile organic compounds of samples were analysed with gaschromatography-mass spectrometry coupled with solid phase microextraction (GC-MS-SPME), using an Agilent Hewlett–Packard 6890 GC gas-chromatograph and a 5970 MSD MS detector (Hewlett–Packard, Geneva, Switzerland) equipped with a Varian (50 m X 0.32 mm X 1.2 μm) fused silica capillary column. In particular, samples (3 g) were placed in 10-ml sterilized vials, added with known amount of 4 methyl- 2-pentanol (Sigma-Aldrich, Steinheim, Germany) as internal standard and sealed by PTFE/silicon septa. The samples were heated for 10 min at 45 °C and then a fused silica SPME fiber covered with 85 μm Carboxen/Polydimethylsiloxane (CAR/PDMS) (Supelco, Steinheim, Germany) was introduced into the headspace for 40 min. Adsorbed molecules were desorbed in the gas-chromatograph for 10 min. The conditions were the same reported by by Montanari et al.

(2018a). Volatile peak identification was carried out by computer matching of mass spectral data with those of compounds contained in the libraries NIST 2005 and 2011.

4.3. RESULTS

4.3.1. ANALYSES OF THE MEAT MIXTURE AND CONTROL OF RIPENING PARAMETERS (pH, WEIGHT LOSSES AND A_W)

The meat mixture used for sausage preparation was analysed immediately before stuffing to determine the microbial counts: LAB 6.20±0.10 log CFU/g, coagulase negative staphylococci (CNS) 6.23±0.12 log CFU/g, total coliforms 2.90±0.10 log CFU/g, fecal coliforms 2.45±0.17 log CFU/g. In addition, pH (5.70±0.02), NaCl content (2.64%±0.01) and glucose concentration (0.49±0.1 g/k) were determined.

Then, the viability of the starter cultures used was assessed, and the results were 9.72±0.30 log CFU/g of dried culture for LAB and 9.72±0.30 log CFU/g of dried culture for CNC. These concentrations, combined with the dilution used during the meat mixture preparation, determined a LAB starter culture concentration of 5.5 log CFU/g and a CNC starter culture concentration of 5.5 log CFU/g. Considering the total counts, the ratio between autochthonous LAB (already present in the raw meat) and starter LAB was approx. 4. Similarly, the ratio between autochthonous CNC (already present in the raw meat) and starter CNC was approx. 4.

The inoculation of selected *Penicillium nalgiovense* strain determined a faster and more uniform colonization (moulds already grew after 3 days, during the drying phase), as shown in Figure 4.1.

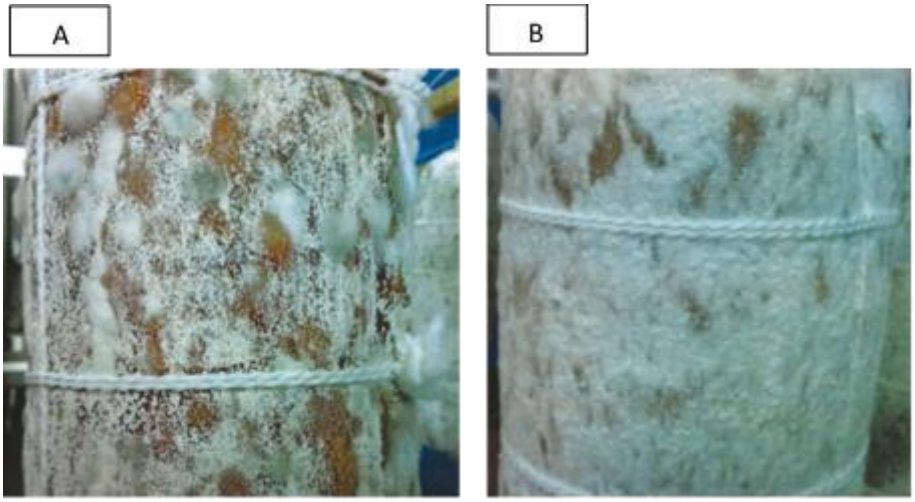

Figure 4.1: Mould growth in Ventricina after 30 days of ripening. On the left (A) the sample with wild moulds and on the right (B) the sample inoculated with *P. nalgiovense*.

During drying and ripening, the pH, weight losses and a_w were monitored. Regarding pH, measurements were carried out in two different parts of the sausages: the outer section (considering the 3 cm of the products immediately below the casing) and the inner one. In Figure 4.2 the pH values during the 100 days ripening period are reported. No significant differences in relation to the use of selected mould starter culture were observed in the inner part (Figure 4.2a). The initial pH value of 5.7 decreased down to 4.75 at the end of fermentation (approx. 7 days) in the inner section after which it constantly increased to reach, after 100 days, a value close to the initial one (5.7). A different behavior characterized the outer part (Figure 4.2.b) in which the pH reduction was slightly lower, followed by a rapid increase (pH 5.3 after 28 days) and then it continued to increase. At the end of ripening (100 days) the pH of the outer part was higher than in the inner part and it was significantly higher in the samples without the addition of *P. nalgiovense* (6.1 vs. 5.9).

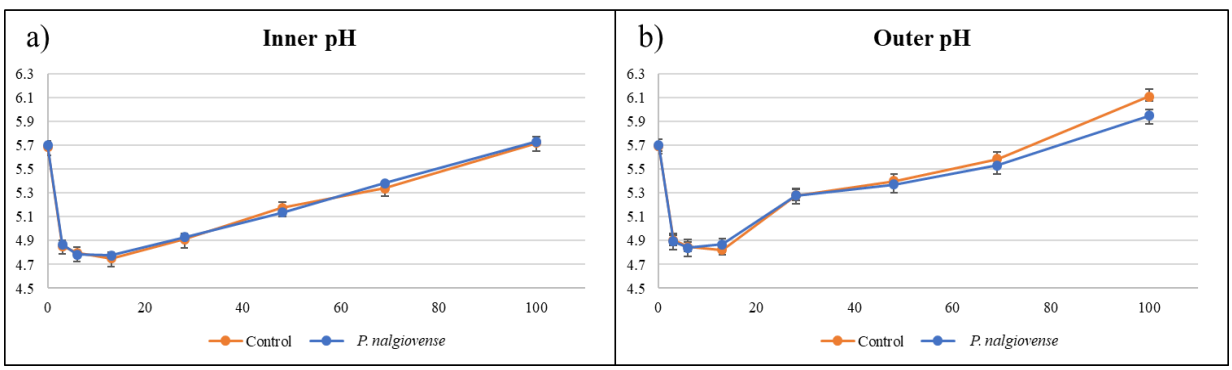

Figure 4.2.: pH values during the 100 days ripening period in the inner part (a) and in the outer section (b) of the two products.

The weight losses (Figure 4.3.) did not show significant differences in relation to mould inoculation and at the end of ripening reached a percentage of approx. 37%. By contrast, a_w resulted higher in the sausages not inoculated with *P. nalgiovense* after 14 days and remained higher throughout all the ripening period and only after 100 days the two sausages showed similar values (approx. 0.92) (Figure 4.4b).

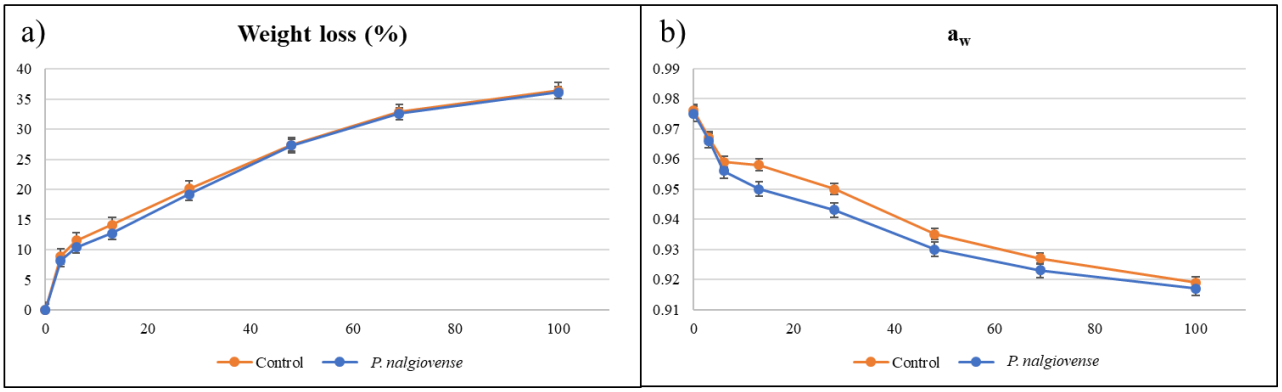

Figure 4.3.: Measurement of weight loss (a) and a_w (b) during the 100 days ripening period of the two products.

4.3.2. MICROBIOLOGICAL ANALYSES OF THE RIPENED SAUSAGES

The periodic controls carried out during ripening revealed after 45 days an olfactive defect in the sausages obtained without the addition of *P. nalgiovense*. It consisted in the presence of off-odours (sweaty, pungent) and it increased during ripening. In addition, these sausages were characterized by the formation of crust on the outer side. At the end of ripening the defects were perceivable also in the product with *P. nalgiovense* but with a much lower impact.

For these reasons, the microbial population present in the inner and in the outer part of sausages were analysed (Table 4.1). LAB were the dominant group with counts higher than 8 log CFU/g in all the samples. In the outer part the counts were higher but not significantly. The same trend was observed for CNS but in this case the differences were significant and reached levels higher than 7 log CFU/g only in the outer sections. In any case, the differences were not significant in relation to the type of mould grown on the casings. At the end of ripening both enterobacteria and pseudomonads were below the detection limits, while enterococci were detected in low amount (about 2 log CFU/g) only in the inner part.

In the same table also the lactic and acetic acid concentrations are reported. Relevant differences were observed between inner and outer sections. Lactic acid showed a similar concentration in both the inner samples (higher than 11 g/kg), while significant differences were observed for the outer part (7.73 g/kg in the presence of *P. nalgiovense* vs. 6.01 g/kg with the wild moulds. Also acetic acid presented higher concentrations in the inner samples (2.01-2.32 g/kg vs. 1.47-1.60 g/kg) but without difference in relation to the mould grown.

Table 4.1: Microbiological analyses (log CFU/g) and organic acids concentration (g/kg) of the ripened sausages.

Sample	LAB	Staphylococci	CMT	*Enterobacteriaceae*	Enterococci	Pseudomonas	Lactic acid	Acetic acid
Control in	8.36 ± 0.12	6.84 ± 0.16	8.61 ± 0.10	<1	1.78 ± 1.78	<1	11.59 ± 0.17	2.32 ± 0.07
Control out	8.51 ± 0.21	7.29 ± 0.10	8.50 ± 0.14	<1	<1	<1	6.01 ± 0.12	1.47 ± 0.15
***P. nalgiovense* in**	8.20 ± 0.22	6.52 ± 0.30	8.30 ± 0.21	<1	2.05 ± 0.57	<1	11.19 ± 0.19	2.01 ± 0.14
***P. nalgiovense* out**	8.30 ± 0.15	7.14 ± 0.12	8.51 ± 0.25	<1	<1	<1	7.73 ± 0.11	1.60 ± 0.06

4.3.3. METAGENOMIC ANALYSES

A metagenomic analysis was carried out in order to verify the contribute of different microbial populations (including starter cultures) to fermentation and ripening of sausages. A total of more than 800 amplicon sequence variants (ASV) were detected. In the Table 4.2 only the ASV which reached a concentration higher than 0.5% in at least one of the samples are reported. In addition, the metagenomic analysis was carried out for both sausages (with wild moulds or inoculated with *P. nalgiovense*) in the inner and in the outer part. Lactobacilli were identified at group level, as described by Salvetti et al. (2018). Several differences were observed regarding both the presence of mould and the inner/outer part of sausages. Among the most important species, *Staphylococcus xylosus*, added as starter culture, was present in extremely different proportion in the inner and outer part of the sausages with wild moulds (29.3 and 78.3% of ASV). By contrast, in the sausages inoculated with *P. nalgiovense* the presence of this species was higher in the inner (48.6%) rather than in the outer part (20.5%). Other staphylococci species were present at lower

concentrations and a higher presence of *S. carnosus* in the outer part of both the sausages was observed.

The composition of the lactobacilli demonstrated the failure of the starter cultures to dominate the environment. In fact, lactobacilli belonging to *Lactobacillus sakei* group (presumably the *L. sakei* strain used as starter culture) represented the 26% of ASV in the inner part of Ventricina with wild moulds, while its concentration was even lower in the other samples and especially in the outer part of the sausage not inoculated with *P. nalgiovense*, in which it represented only the 1.3% of ASV. Other lactobacilli species prevailed, especially in the sausages inoculated with *P. nalgiovense*, and in particular LAB belonging to the *Lactobacillus plantarum* group and heterofermentative LAB belonging to the *Lactobacillus buchneri* group. In both cases, their presence was higher in the outer than in the inner part of Ventricina. Lower ASV belonging to the *Lactobacillus alimentarius* and *Lactobacillus casei* groups were also found.

Among LAB, also other species were detected, such as *Pediococcus pentosaceus*, *Tetragenococcus koreensis*, *Lactococcus lactis*, *Weissella* sp. and *Streptococcus* sp.

Other Gram-positive bacteria detected especially in the inner part of sausages without differences depending on the mould growth belonged to the *Propionibacteriaceae* (*Cutibacterium* sp.) and *Corynebacteriaceae* (*Corynebacterium accolens*). A relevant presence (1.5% of ASV) of *Actinomyces* sp. was observed only in the outer part of the sausage obtained without the inoculum of *P. nalgiovense.*

Few Gram-negative bacteria were present according to the metagenomic analysis carried out at the end of ripening. Low concentrations of Bacteroidetes (*Prevotella conceptionensis* and *Cloacibacterium normanense*) were observed in the inner part of both sausages, while among Proteobacteria *Pseudomonas* sp. prevailed in the outer part of the sausages.

The differences in the composition of the microbial communities is also reported as relative frequency in the histograms in Figure 4.4.

Table 4.2: Results of metagenomic analyses (only the ASV which reached a concentration higher than 0.5% in at least one of the samples are reported).

				Identification	**Control in**	**Control out**	***P. nalgiovense* in**	***P. nalgiovense* out**
Actinobacteria	Actinobacteria	Propionibacteriales	*Propionibacteriaceae*	*Cutibacterium* sp.	2.88	0.09	2.73	0.15
Actinobacteria	Actinobacteria	Actinomycetales	*Actinomycetaceae*	*Actinomyces* sp.	0.01	1.48	0.00	0.02
Actinobacteria	Actinobacteria	Corynebacteriales	*Corynebacteriaceae*	*Corynebacterium accolens*	1.34	0.03	1.95	0.07
Actinobacteria	Actinobacteria	Corynebacteriales	*Corynebacteriaceae*	*Corynebacterium* sp.	0.00	0.61	0.18	0.10
Bacteroidetes	Bacteroidia	Bacteroidales	*Prevotellaceae*	*Prevotella conceptionensis*	0.29	0.00	0.75	0.00
Bacteroidetes	Bacteroidia	Flavobacteriales	*Weeksellaceae*	*Cloacibacterium normanense*	0.13	0.00	0.74	0.09
Firmicutes	Bacilli	Bacillales	*Bacillaceae*	*Anoxybacillus flavitermus*	0.00	0.00	1.10	0.05
Firmicutes	Bacilli	Bacillales	*Staphylococcaceae*	*Staphylococcus xylosus*	29.30	78.34	48.58	20.49
Firmicutes	Bacilli	Bacillales	*Staphylococcaceae*	*Staphylococcus carnosus*	0.95	4.42	0.77	1.10
Firmicutes	Bacilli	Bacillales	*Staphylococcaceae*	*Staphylococcus saccharolyticus*	0.72	0.00	0.69	0.03
Firmicutes	Bacilli	Bacillales	*Staphylococcaceae*	*Staphylococcus hominis*	0.18	0.77	0.04	0.10
Firmicutes	Bacilli	Lactobacillales	*Enterococcaceae*	*Tetragenococcus koreensis*	0.07	1.68	0.39	0.08
Firmicutes	Bacilli	Lactobacillales	*Lactobacillaceae*	*L. plantarum* group	15.15	5.20	13.37	34.25
Firmicutes	Bacilli	Lactobacillales	*Lactobacillaceae*	*L. sakei* group	25.98	1.29	7.90	12.37
Firmicutes	Bacilli	Lactobacillales	*Lactobacillaceae*	*L. buchneri* group	9.63	2.53	6.22	20.76
Firmicutes	Bacilli	Lactobacillales	*Lactobacillaceae*	*L. coryniformis* group	2.45	0.46	2.36	4.00
Firmicutes	Bacilli	Lactobacillales	*Lactobacillaceae*	*L. alimentarius* group	1.97	0.51	3.03	0.82
Firmicutes	Bacilli	Lactobacillales	*Lactobacillaceae*	*L. casei* group	0.86	0.06	0.00	0.78
Firmicutes	Bacilli	Lactobacillales	*Lactobacillaceae*	*Pediococcus pentosaceus*	1.14	0.08	0.26	0.38
Firmicutes	Bacilli	Lactobacillales	*Leuconostocaceae*	*Weissella* sp.	0.19	0.89	0.64	1.88
Firmicutes	Bacilli	Lactobacillales	*Streptococcaceae*	*Streptococcus* sp.	1.56	0.01	0.81	0.03
Firmicutes	Bacilli	Lactobacillales	*Streptococcaceae*	*Lactococcus lactis*	0.69	0.05	0.06	1.10
Firmicutes	Clostridia	Clostridiales	Family_XI	*Peptoniphilus rhinitidis*	0.34	0.01	1.28	0.00
Proteobacteria	Alphaproteobacteria	Caulobacterales	*Caulobacteraceae*	*Caulobacter segnis*	0.20	0.01	0.83	0.06
Proteobacteria	Gammaproteobacteria	Betaproteobacteriales	*Burkholderiaceae*	*Hydrogenophaga intermedia*	0.00	0.00	1.14	0.00
Proteobacteria	Gammaproteobacteria	Pseudomonadales	*Pseudomonadaceae*	*Pseudomonas* sp.	3.32	0.07	3.86	0.11

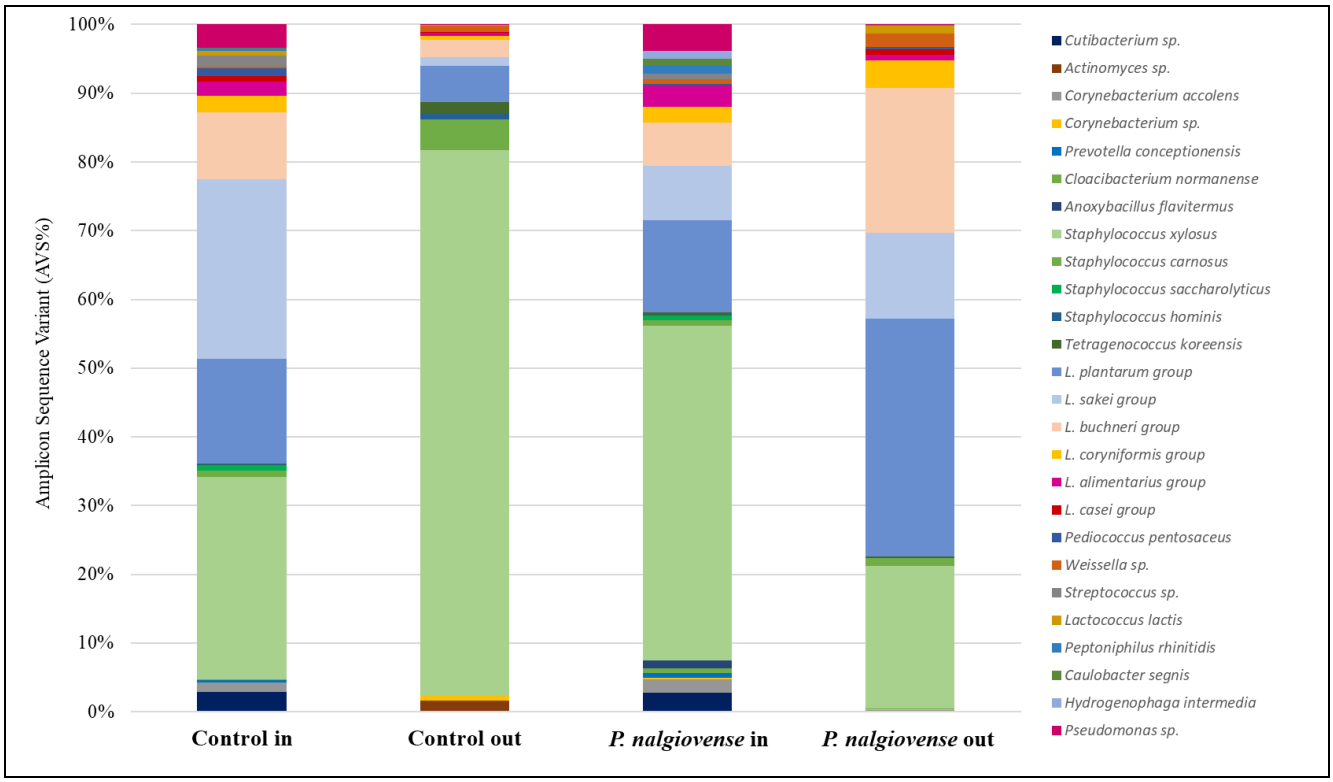

Figure 4.4.: Differences in the composition of the microbial communities in terms of relative frequency.

4.3.4. BIOGENIC AMINES (BA) DETERMINATION

The BA amount resulted extremely high in both the conditions (Table 4.3.). In general, the BA content was higher in the inner part of sausages, due to the major activity of decarboxylases in the presence of low oxygen concentration and the response to acid stress determined by the lower pH inside the sausage. In general, all the BAs had lower concentration in the inner part of the sausages inoculated with *P. nalgiovense*, while an opposite situation characterized the outer part. Histamine, the most dangerous BA, reached worrying concentrations (approx. 200 mg/kg) in the internal part of both the samples. There are no legal limit for BAs in sausages, but a limit of 200 mg/kg of this amine is included by many international Institutions (FDA, EFSA) for some fishery products. The concentration of tyramine was very similar to histamine (about 200 mg/kg in all the samples). Relevant differences between outer and inner part were observed for putrescine and cadaverine. Putrescine, deriving from ornithine decarboxylation, showed concentrations higher than 300 mg/kg inside the sausage while in the external part the values were 211.1 and 246.6 mg/kg in the sausage with the wild moulds and in the sausages inoculated with *P. nalgiovense*, respectively. A similar behavior characterized cadaverine (produced by lysine

decarboxylation), with concentrations ranging between 551.7 and 597.0 mg/kg in the inner sausages and between 270.8 and 333.3 mg/kg in the outer sausages. 2-phenylethylamine was accumulated in low amounts (lower than 20 mg/kg) in all the samples.

Sample	Histamine	Tyramine	Putrescine	Cadaverine	2-phenylethylamine	Total
Control in	223.6 ± 14.6	226.3 ± 14.5	351.5 ± 20.1	597.0 ± 21.9	15.2 ± 3.2	1413.6
Control out	151.2 ± 13.5	181.5 ± 11.7	211.1 ± 12.3	270.8 ± 13.2	10.7 ± 4.0	825.3
***P. nalgiovense* in**	195.9 ± 8.9	200.5 ± 15.3	314.6 ± 18.5	551.7 ± 15.7	16.4 ± 4.8	1279.1
***P. nalgiovense* out**	180.8 ± 10.5	193.6 ± 6.6	246.6 ± 14.1	333.3 ± 10.2	12.2 ± 2.6	966.2

Table 4.3. Biogenic amines content.

The presence of such relevant amounts of BA is a further indication of the failure of the starter cultures in colonizing the meat environment. In fact, one of the criteria in the selection of microorganisms for food fermentation is the inability to produce BA. In addition, the species *L. sakei* is characterized by the lack of amino acid decarboxylase activity, with the exception of some strains able to produce small amounts of putrescine as result of the arginine deiminase (ADI) pathway (Montanari et al., 2018; Rimaux et al., 2012). Thus, other bacteria are involved in the decarboxylase activities. Regarding tyramine, it is well known that LAB are the most efficient producers of this BA (Marcobal et al., 2012). Given the lack of enterococci (the most active in tyrosine decarboxylation), other LAB, among which many lactobacilli (such as *L. brevis*, *L. fermentum*, *L. plantarum*, *L. buchneri*, *L. casei*) can be responsible for its accumulation (Barbieri et al., 2019; Marcobal et al., 2012) Similarly, histamine can be produced by several LAB species, such as *L buchneri*, *L. plantarum* and *L. casei* (Barbieri et al., 2019). The great production of aliphatic polyamines (cadaverine and putrescine) observed in these sausages is usually associated with a relevant growth of Gram negative bacteria, and, in particular, of *Enterobacteriaceae* (Suzzi and Gardini, 2003). According to the metagenomic analysis, the presence of AVS attributable to this Family is rather limited. Thus, the production could be again attributed to LAB. In fact, the ability to produce great amounts of cadaverine and putrescine has already been observed in *L. plantarum* (Alan et al., 2018) and *L. brevis* (Lee et al., 2019). Alternatively, an initial growth of Gram negative bacteria at the beginning of fermentation could be responsible for their accumulation, taking into account that the

decarboxylases can be active also outside the cells following death and lysis of bacteria (Gardini et al., 2016). In this case, it is possible that the DNA of these cells has been used by other bacteria (mainly LAB) during the 100 days ripening period for using the nucleoside as carbon source in a limiting nutritional environment, as demonstrated in *L. sakei* (Rimaux et al., 2011).

4.3.5. AROMA PROFILE OF SAUSAGES

According to the producer controls, the sausages not inoculated with *P. nalgiovense* presented an organoleptic defect during ripening, perceivable after about 60 days and consisting in the presence of off-odours (sweaty, putrefaction). Thus, the aroma profile of sausages was analysed using a SPME-GC-MS protocol already described for sausages (Montanari et al., 2018a). The results are reported in Table 4.4 and in Figure 4.5., in which the compounds are divided into classes.

Table 4.4: Aroma profile of sausages.

Compounds	Control out	Control in	*P. nalgiovense* out	*P. nalgiovense* in
Nonanal	1.47	2.48	2.21	1.20
Decanal	0.26	0.17	1.26	0.69
Benzaldehyde	4.60	3.68	1.84	1.59
Benzeneacetaldehyde	44.18	35.88	43.11	39.55
Aldehydes	**50.52**	**42.21**	**48.42**	**43.02**
Ethyl alcohol	46.59	41.13	41.48	35.26
2-butanol	29.89	24.53	22.82	21.09
1-propanol	54.81	48.93	53.48	52.37
Benzyl alcohol	4.16	3.08	2.28	2.29
Phenylethyl alcohol	10.66	10.25	9.66	14.20
Alcohols	**146.11**	**127.92**	**129.72**	**125.21**
2-butanone	22.07	12.70	17.93	14.43
3-Penten-2-one, 4-methyl	3.13	1.92	3.05	2.40
2-Nonanone	0.84	0.25	0.45	0.00
Ketones	**26.04**	**14.87**	**21.43**	**16.82**
Ethyl Acetate	2.75	3.00	2.76	2.76
Propanoic acid, ethyl ester	1.79	1.96	1.59	1.92
n-propyl acetate	4.14	4.85	4.60	5.47
Propanoic acid, propyl ester	5.09	4.57	4.35	4.35
Octanoic acid, ethyl ester	2.53	2.39	2.68	2.33
Octanoic acid, propyl ester	2.49	2.39	2.54	2.88
Esters	**18.80**	**19.16**	**18.51**	**1971**

Acetic acid	113.11	139.97	85.60	108.73
Propanoic acid	26.71	34.25	20.01	27.40
Butanoic acid	5.15	6.08	4.07	6.14
Butanoic acid, 3-methyl	13.56	14.59	0.96	0.63
Hexanoic acid	1.60	5.73	2.07	3.38
Octanoic acid	8.31	10.57	7.65	8.86
n-decanoic acid	6.63	7.77	13.05	11.30
n-Hexadecanoic acid	59.63	70.53	61.45	63.74
Acids	**234.69**	**289.49**	**194.85**	**230.19**

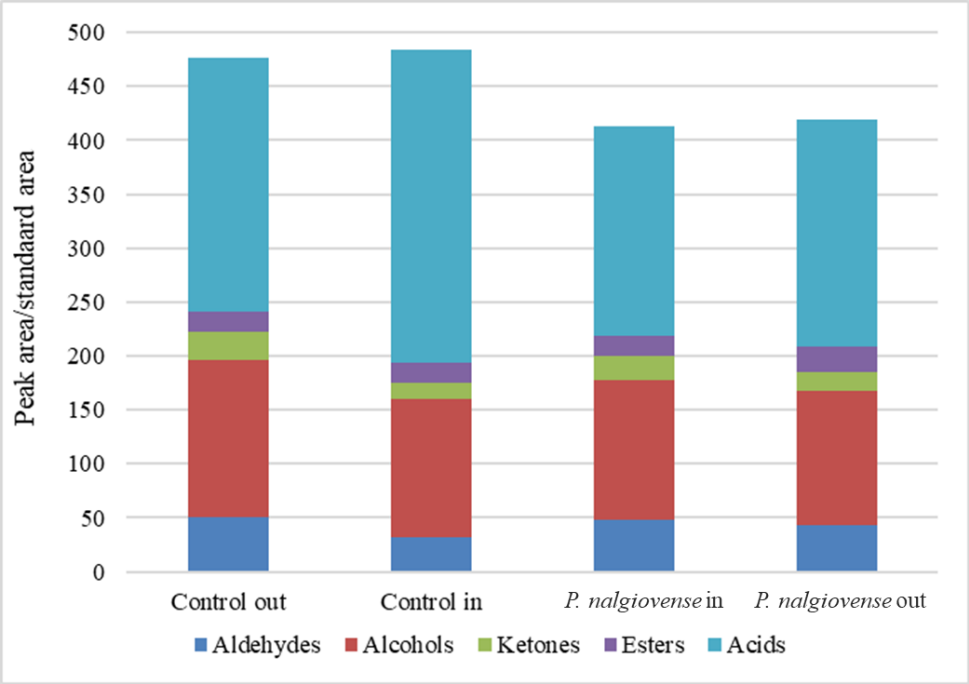

Figure 4.5: Classes of aroma compounds in each sample.

Among aldehydes, benzeneacetaldeyde prevailed but the amounts of this molecule deriving from phenylalaline metabolism (Tabanelli et al., 2012) did not significantly differ in relation to the section of the sausage or the inoculum of *P. nalgiovense*. Also benzaldehyde derives from the same amino acid and it was present in lower amounts, but with higher concentration in the sausages ripened with wild moulds. Scarce was the presence of aldehydes deriving from lipid oxidation. In fact, only small quantities of nonanal and decanal were detected. Usually, the presence of these aldehydes is much higher in Mediterranean fermented sausages (Montanari et al., 2016, 2018). The lower amounts observed here may be due to the long ripening times (which can be responsible for further transformation of these molecules) or to the low fat content of this type of sausages and to the big size of fat cubes which reduce the total surface exposed to the oxidation.

Alcohols were represented mainly by ethanol, with higher content in the outer part of sausages and in the samples not inoculated with *P. nalgiovense*. The presence of ethanol can be attributed to several pathways and together with acetic acid, may result from LAB metabolism of lactate (von Wright and Axelsson, 2011). Together with ethanol, 1-propanol was the most important alcohol present in both sausages. Usually this alcohol is present in proportions much lower than those observed here. It may be produced by reduction of the corresponding aldehyde (pentanal), which can be formed by oxidative reactions (Ordóňez et al., 1999). Alternatively, some LAB (such as *L. reuteri*) can produce this molecule as result of secondary pathways addressed to NADH regeneration (Gänzle, 2015).

Also 2-butanol, resulting from the reduction of 2-butanone, presented a similar behavior. Benzyl alcohol and phenethyl alcohol are the results of reduction of benzaldehyde and benzeneacetaldehyde, respectively.

The presence of ketones was low and mainly constituted by 2-butanone. High amount of this ketone characterized Italian sausages with a large diameter (Montanari et al., 2018a). The presence of methyl ketones in sausages may derive from β-ketoacids produced during β-oxidation carried out by moulds and staphylococci (Lorenzo et al., 2016; Olivares et al., 2011). Nevertheless, lactobacilli can produce 2-butanone starting from diacetyl through the action of a diol dehydratase (Speranza et al., 1997). However, differently from similar products, the presence of diacetyl or acetoin was never detected in these sausages.

Several esters were detected, even if these compounds accounted for a limited proportion of volatile molecules. They were mainly ethyl or propyl esters and no significant differences were observed either in relation to mould inoculation or to the section of sausage considered. Staphylococci are usually the bacteria responsible for the major esterase activity in sausages (Flores and Olivares, 2015; Sánchez-Mainar et al., 2017).

The main differences were found for acids. Acetic acid was present in higher proportion in the sausages without selected mould inoculation and in the inner part of sausages. Similar behaviors were observed for propanoic acid and octanoic acid. The major differences concerned the presence of 3-methyl-butanoic (isovaleric) acid. According to Smit et al. (2004) this acid is a result of leucine metabolism, which is firstly deaminated and then the resulting α-ketoisocaproic acid can be transformed into isovaleric acid following two ways, the first involving the action of a decarboxylase and the second a α-ketoacid dehydrogenase

(oxidative decarboxylation). This second way allows the production of ATP and may be advantageous for LAB in an environment poor in fermentable substrates such as a sausage after several weeks of ripening. The presence of isovaleric acid in fermented sausages is well documented (Gianelli et al., 2011). It is characterized by a high odour activity value and is accumulated mainly at the end of the ripening process (Olivares et al., 2009). Its contribution to the overall sausage aroma depends on its concentration and has been described as characterized by mild sweet of fruity notes (Carballo, 2012) but higher concentration may result in cheese, feet and dirty socks smell (Olivares et al., 2019). The possibility to produce isovaleric acid starting from leucine has already been described in *Carnobacterium piscicola* (Larrouture-Thiveyrat and Montel, 2003), *Staphylococcus carnosus* (Larrouture-Thiveyrat et al., 2000; Masson et al., 1999) and also from aspergilli and penicillin (Coll and Leal, 1972). In particular, the products of leucine metabolism have been considered as metabolic markers for staphylococci activity in fermented meat (Stavropoulou et al., 2015; Stahnke, 1995).

4.4. CONCLUSIONS

The defects presented by the sausages considered in this study have to be attributed in first instance to a failure of the action of LAB and staphylococci added as starter cultures for driving the fermentation process. The meat used for the production was characterized by a high microbial count and the starter cultures were not able to colonize the environment reducing or inhibiting the growth of the wild microbial communities, as demonstrated by metagenomic analyses. This failure determined a spontaneous fermentation resulting in a relevant accumulation of biogenic amines, which was independent of the presence of the selected strains of mould. Nevertheless, the presence of *P. nalgiovense* determined a different behavior in the lowering of a_w during ripening and a different composition of the microbial communities analysed at the end of ripening. These differences were particularly remarkable in the outer part of sausages in which staphylococci play a major role and in which a considerable presence of *Actinomyces* sp. was observed. The results of population dynamics caused a different aroma profile of the sausages: in particular, the absence of *P. nalgiovense* was responsible for the production of high amounts of isovaleric acid, an

important contributor to the off odour characterizing the Ventricina obtained with wild moulds.

The conclusion of this work is that the addition of starter culture alone is necessary but not sufficient to guarantee the obtaining of sausages with acceptable quality level. The use of starter culture needs to be modulated in relation to other production parameters. In particular, the initial microbial meat contamination influences the possibility of the selected bacteria to colonize the environment bringing to safety (biogenic amine content) and organoleptic (off odours) concerns.

CHAPTER 5

PHENOTYPIC DIVERSITY OF *LACTOBACILLUS SAKEI* STRAINS

5.1. INTRODUCTION

Lactobacillus sakei is a lactic acid bacteria (LAB) highly adapted to grow in meat environments in which it can outcompete undesired microorganisms, including pathogenic species (Chaillou et al., 2013). For this reason, it is often responsible for natural fermentation of dry fermented sausages (Hugas et al., 1993). Because of this aptitude, selected strains of this species are widely used as starter cultures in meat fermentation together with strains belonging to the species *L. curvatus*, *Pediococcus pentosaceus* and *P. acidilactici* (Champomier-Vergès et al., 2001; Hammes and Hertel, 1998). The main energy sources are sugars: hexose fermentation is homolactic while pentoses (such as ribose) are fermented through the heterolactic pathway (McLeod et al., 2008; Rimaux et al., 2011b). Nevertheless, the ability of the species to dominate the microbiota of fermented sausages for several weeks, when the hexoses are depleted after few days from the production, underlines its ability to use other substrates to obtain energy for growth and survival (Cocconcelli and Fontana, 2010). Within the species, two subspecies are recognized (Torriani et al., 1996), *L. sakei* ssp. *sakei* and *L. sakei* ssp. *carnosus*, which differ for the presence of specific soluble cell proteins. However, Chaillou et al. (2013) recently suggested that members of this species derived from three ancestral lineages determined by independent selection scenarios.

L. sakei is characterized by a wide genetic and phenotypic diversity. In the last years, its genome has been sequenced and a high variability in the dimension has been observed. The mean genome size was 2020 kb with a variation of about 25% (from 1814 to 2309 kb) (Chaillou et al., 2005; Chaillou et al., 2009).

The genome analysis evidenced some specific traits of this species, which can explain its high adaptation to meat environment (Claesson et al., 2007). Studies have been carried out on the ability to catabolize arginine (Rimaux et al., 2011a; Rimaux et al., 2012), the purine nucleoside metabolism (Rimaux et al., 2011b) and the high adaptability to some adverse environment conditions such as cold, oxidative and high salt stresses (Belfiore et al., 2013; Duhutrel et al., 2010; Guilbaud et al., 2012). In particular, the utilization of the ribose present in nucleosides and the activation of the arginine deiminase (ADI) pathway can be additional energy sources giving a competitive advantage in matrices with low fermentable sugar concentration (McLeod et al., 2017).

Amino acids play a key role in explaining *L. sakei* survival and growth in meat. The species is auxotrophic for all amino acids except aspartic and glutamic acids, which can be obtained by the deamination of asparagine and glutamine, respectively (Chaillou et al., 2005). The absence of the metabolic pathways for amino acid synthesis and the absence of transaminases are a result of the adaptation of *L. sakei* to meat, a substrate extremely rich in these molecules, which can be up-taken as free amino acids or short peptides (Sinz and Schwab, 2012). In addition to their role in protein synthesis, amino acid can be involved in other extremely important pathways for the overall cell metabolism. They can in fact contribute to energy generation (Fernández and Zúñiga, 2006) as well as to the production in sausages of aroma compounds or undesired substances such as biogenic amines (Smid and Kleerebezem, 2014; Suzzi and Gardini, 2003).

The genomic variability of *L. sakei* has already been demonstrated. Nevertheless, as observed by Sinz et al. (2013), the metabolic activities of a microorganism (or of a species) can be only hardly deduced from the genome and additional biochemical efforts are needed to evaluate the phenotypic potential of strains of industrial interest.

In this work, the growth performances of six *L. sakei* strains in relation to temperature and salt concentration were modelled. Moreover, in order to understand how a primary energy source could affect the overall strain metabolism, the strains were inoculated in a defined medium (DM) containing 20 free amino acids and added or not with sugars (glucose or ribose). After 24 h of incubation, analyses were carried out in order to quantify the cell number, pH and the amounts of amino acids in the DM, as well as the organic acid produced (L-lactate, D-lactate, acetate, formate). In addition, also C4 volatile compounds produced by the strains (diacetyl and acetoin) were evaluated.

5.2. MATERIALS AND METHODS

5.2.1. STRAINS

Six *L. sakei* strains were employed in this work. The type strain DSMZ 20017^{t} (DSMZ, Braunschweig, Germany), the collection strain DSMZ 6333, isolated form vacuum-packaged pork meat, a strain (Chr82) provided by Chr. Hansen (Parma, Italy) and three strains belonging to collection of Dipartimento di Scienze e Tecnologie Agroalimentari (University of Bologna). In particular, BR3 and TA13 were isolated from spontaneous

fermented pork sausages produced in Emilia-Romagna region while the strain CM3 were isolated from dried camel meat produced in Algeria (Gozzi et al., 2017).

The *L. sakei* strains were maintained in de Man Rogosa and Sharp (MRS) medium (Oxoid, Basingstoke, UK) with 20% (w/v) glycerol at -80°C until usage. Before the experiments, strains were pre-cultivated twice in MRS medium for 24 h at 30°C.

5.2.2. GROWTH MODELLING AT DIFFERENT TEMPERATURE AND SALT CONCENTRATION

The *L. sakei* strains were inoculated in MRS (initial concentration approx. 4 log cfu/ml) and their growth in relation to temperature and NaCl concentration was monitored through the variation of optical density at 600 nm (OD_{600}), measured with a UV–VIS spectrophotometer, 6705 UV-Vis (Jenway, Stone, UK). Medium pH at the end of growth was also measured (pH meter Basic 20, Crison, Modena, Italy) and the acidification activity was expressed as pH decrease with respect to the initial value (about 6.5). In particular, the effect of temperature was monitored by incubating the cultures from 5 to 40°C (with a step of 5°C) while the effect of NaCl was determined at 30°C adding 0, 2, 4, 6, and 8% (w/v) of NaCl to MRS before sterilization.

The OD_{600} data were fitted with the Gompertz equation as modified by Zwietering et al. (1990).

$$y = Ae^{-e^{\left[\left(\frac{\mu max e}{A}\right)(\lambda - t)+1\right]}}$$

where y is the OD_{600} at time *t*, *A* represents the maximum OD_{600} value reached, μ_{max} is the maximum OD_{600} increase rate in exponential phase and λ is the lag time.

5.2.3. INOCULUM IN DEFINED MEDIUM (DM)

After a pre-growth in MRS medium at 30°C for 24 h, the *L. sakei* strains were cultured in a modified MRS containing 25 mM of glucose or 25 mM of ribose as fermentable carbohydrates, for 24 h at 30°C. In the case of MRS added with ribose, according to the observation of McLeod et al. (2008), a small amount of glucose (1 mM) was also used. After growth, the cells were collected in the early stationary phase by centrifugation (10000 × g for 10 min), washed twice with physiological solution (0.9%, w/v NaCl) and re-

suspended at a concentration of about 9 log cfu/ml in different defined media (DM), sterilized by filtration. The composition of DM (adapted from Lauret et al., 1996), containing defined amounts of amino acids (added at a concentration of 0.2 g/L), vitamins and growth factors, is reported in Table 5.1. Cells pre-grown in the presence of glucose were re-suspended in DM containing glucose 25 mM (25-G) or without sugar (0-G). Similarly, cells pre-grown in the presence of ribose were re-suspended in DM containing ribose 25 mM (25-R) or without sugar (0-R) (Figure 5.1.). Samples were incubated at 30°C for 24 h. Three independent samples for each condition were analysed.

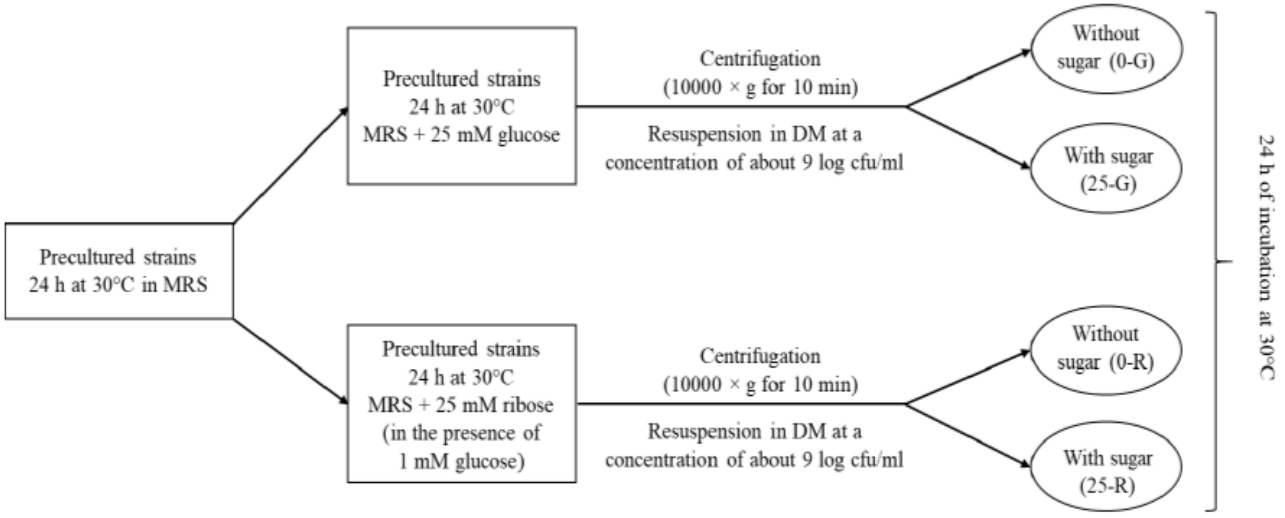

Figure 5.1.: Experimental plan for defined medium (DM) trial.

Components	Concentration	Components	Concentration
Macro components	**mM**	**Amino acids**	**mM**
KCl	10.06	Glutamic acid (glu)	1.36
$MnSO_4$	0.05	Aspartic acid (asp)	1.50
$MgSO_4$	1.66	Alanine (ala)	2.25
Na_2HPO_4	12.33	Arginine (arg)	1.15
Tween 80	1.65	Asparagine (asg)	1.51
		Cysteine (cys)	1.65
Vitamins	**µM**	Glutamine (glm)	1.37
Thiamine HCl	3.0	Glycine (gly)	2.66
Folic acid	0.5	Histidine (his)	1.29
Riboflavin	2.7	Isoleucine (ile)	1.53
Calcium panthotenate	4.6	Leucine (leu)	1.53
Nicotinic acid	8.1	Lysine (lys)	1.37
Pyridoxal	3.0	Methionine (met)	1.34
p-aminobenzoic acid	2.9	Phenylalanine (phe)	1.21
		Proline (pro)	1.74
Nucleotides	**mM**	Serine (ser)	1.90
Adenine	0.037	Threonine (thr)	1.68
Guanine	0.046	Ttryptophan (try)	0.98
Uracil	0.089	Tyrosine (tyr)	1.10
		Valine (val)	1.71

Table 5.1.: Defined medium (DM) composition. The concentration of the components was adapted from Lauret et al., 1996.

5.2.4. SURVIVAL ANALISYS AND DM PH

DM samples were incubated at 30°C and, after 24 h, cell survival was assessed by plate counting in MRS agar (Oxoid) incubated for 48 h at 30°C. In addition, pH was monitored using a pH meter Basic 20 (Crison).

5.2.5. DIACETYL, ACETOIN AND ETHANOL DETERMINATION

Volatile organic compounds of samples were monitored, after 24 h of incubation at 30 °C in 10 mL sterilized vials sealed with PTFE/silicon septa, using a gas-chromatography-mass spectrometry coupled with solid phase microextraction (SPME-GC-MS). An Agilent Hewlett-Packard 6890 GC gas-chromatograph, equipped with a MS detector 5970 MSD (Hewlett-Packard, Geneva, Switzerland) and a CP-WAX 52CB 50 m x 0.32 mm x 1.2 µm fused silica capillary column was used (Agilent Technologies, Santa Clara, USA). The

samples were pre-equilibrated for 10 min at 45 °C, after that a fused silica fiber covered by 50/30 μm divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS, StableFlex) (Supelco, Steinheim, Germany) was introduced into the headspace for 40 min. Adsorbed molecules were desorbed in the gas-chromatograph for 10 min. The conditions were the same reported by Montanari et al. (2016). Blanks (empty vials) were injected regularly to monitor possible carry over. An auto-tune of the GC-MS was daily carried out prior to the analysis to ensure optimal GC-MS performance. The volatile compounds were identified by computer matching of mass spectral data with those of compounds contained in NIST 2011 mass spectral library (Scientific Instrument Services, Ringoes, USA). Moreover, for the most important compounds, the mass spectrum identification was confirmed by injection of the pure standards (Sigma-Aldrich, Saint Louis, USA) in the same conditions. The compounds were reported as ratio between each molecule peak area and peak area of the internal standard, 4-methyl-2-pentanol (Sigma-Aldrich), added at a final concentration of 33 mg/kg.

5.2.6. ORGANIC ACIDS AND BIOGENIC AMINE PRODUCTION

The concentration of organic acids in the samples was determined by an HPLC (PU-2089 Intelligent HPLC quaternary pump, UV-VIS multiwavelength detector UV 2070 Plus; Jasco Corp., Tokyo, Japan) and a manual Rheodyne injector with a 20 μL loop (Rheodyne, Rohnert Park, USA), equipped with a Bio-Rad Aminex (Bio-Rad Laboratories, Hertfordshire, UK) HPX-87H column (300x7.8 mm). The analysis was performed in isocratic conditions, using mobile phase H_2SO_4 0.005 M, with rate flow of 0.6 ml/min and temperature of 65°C. The UV detector was set at 210 nm. Chromatographic peaks were identified by comparing retention times with those of standards (Sigma-Aldrich) and quantification was carried out by using the external standard method.

Biogenic amines were detected and quantified according to the HPLC method reported by Bargossi et al. (2015). Under these analytical conditions, the biogenic amines detected were 2-phenylethylamine, putrescine, cadaverine, histamine, tyramine, spermine, spermidine.

5.2.7. AMINO ACIDS QUANTIFICATION

For the variation of amino acid concentrations in DM media, samples were subjected to an AccQ-Fluor Reagent (AQC, 6-aminoquinolyl-N-hydroxysuccinimide carbamate) derivatization (Waters Corp., Milford, USA) according to the manufacturer's protocol. AQC was reconstituted at a final concentration of 10 mM in acetonitrile, included in the AccQ-Fluor Reagent Kit (Waters Corp). Briefly, 10 µL of samples were derivatizated with 70 µL of AccQ-Fluor Borate Buffer (Waters Corp.) and 20 µL of reconstituted reagent. The samples were heated to 55 °C for 10 min. The amino acids content was analysed using an HPLC (PU-1580 Intelligent HPLC pump, Intelligent Fluorescence Detector FP-1520 and Intelligent Sampler AS-2055 Plus, with 10 µl loop; Jasco Corp.). Separation of amino acids was obtained using AccQ-Tag^{TM} column (3.9x150 mm) for amino acid analysis (Waters Corp.). A gradient elution was performed maintaining a column temperature of 30°C and using two mobile phases: A (100 ml of AccQ-Tag Eluent A concentrate (Waters Corp.), diluted 1:10 with H_2O for chromatography (Sigma-Aldrich) and B (60% acetonitrile and 40% H_2O for chromatography) (Sigma-Aldrich) with a flow rate of 1 ml/min. The fluorescence detector was set at excitation wavelength of 250 nm and emission wavelength of 395 nm.

5.2.8. STATISTICAL ANALYSIS

The estimates of parameters of Gompertz equation were obtained using the non linear regression procedure of Statistica for Windows (Statistica 8 software, 2006; StatSoft, Tulsa, U.S.A.). ANOVA was performed using the one-way procedure of Statistica and significant differences between the conditions were evaluated with the LDS test ($p \leq 0.05$). The heat map was obtained in the statistical environment R (R Development Core Team, Vienna, Austria).

5.3. RESULTS AND DISCUSSION

5.3.1. GROWTH MODELLING AT DIFFERENT TEMPERATURE AND SALT CONCENTRATION

The *L. sakei* strains were inoculated in MRS (initial concentration approx. 4 log cfu/ml) and their growth in relation to temperature and NaCl concentration was monitored through the

variation of OD_{600} and modelled with the Gompertz equation (Zwietering et al., 1990). The estimates for the Gompertz parameters are reported in Figure 5.2. and 5.3. The figures report also the pH decrease determined by the strain growth in the different conditions with respect to the initial value of about 6.5. The presence of significant differences among the estimates of Gompertz parameters in relation to temperature and salt were tested with one-way ANOVA.

Regarding temperature (Figure 5.2.), all the strains grew between 5 and 35°C but none was able to grow at 40°C. The values of standard deviation underlined a great variability in the strain performances, especially at the lower temperatures. At 5°C the coefficients of variation (CV) observed for *A*, μ_{max} and λ were 61.4, 72.5 and 34.4%, respectively. The CV was lower for higher temperatures, but it remained rather high, especially for μ_{max} and λ. The best mean growth performances were observed for A and λ between 25 and 35°C without significant differences according to ANOVA ($P > 0.05$). On the contrary, the best performance for μ_{max} was recorded at 35°C, even if without significant differences with the value in the sample incubated at 30°C. Also, pH decrease was higher at 30°C but without significant differences with the samples at 35 and 25°C. Generally, *L. sakei* DSMZ 20017^t determined the lower pH decrease while the strain Chr82 induced the higher acidification, especially at 5°C.

A wide variability was observed also in relation to NaCl and the standard deviations increased with the increase of its concentration (Figure 5.3.). However, all the strains were able to grow at the highest amount tested (8%) even if with a high variability in their performance, *i.e.* with CV of 21.5, 41.8 and 62.0% for *A*, μ_{max} and λ, respectively. The pH decrease reflected the results observed for the parameter *A*. Also in the case of the presence of 8% NaCl, pH decreased of 1.0-1.5 units.

These variations of *L. sakei* strains in growth characteristics and acidification on MRS has already been evidenced by McLeod et al. (2008). Ammor et al. (2005) underlined the presence of relevant phenotypic differences within 36 *L. sakei* strains which regarded the growth performances and the final pH reached by the strains grown on SB medium. The same Authors revealed a noteworthy variability of the same strains in relation to the growth temperature (however, no strain was able to grow at 0 and 45°C), pH (few strains grew at 3.9) and NaCl concentration (no strain grew at NaCl 10% and about one third grew at

6.5%). By contrast, Drosinos et al. (2007) found that 7.7 % of *L. sakei* strains from Greek sausages were able to grow at 45°C and in the presence of 10% of NaCl.

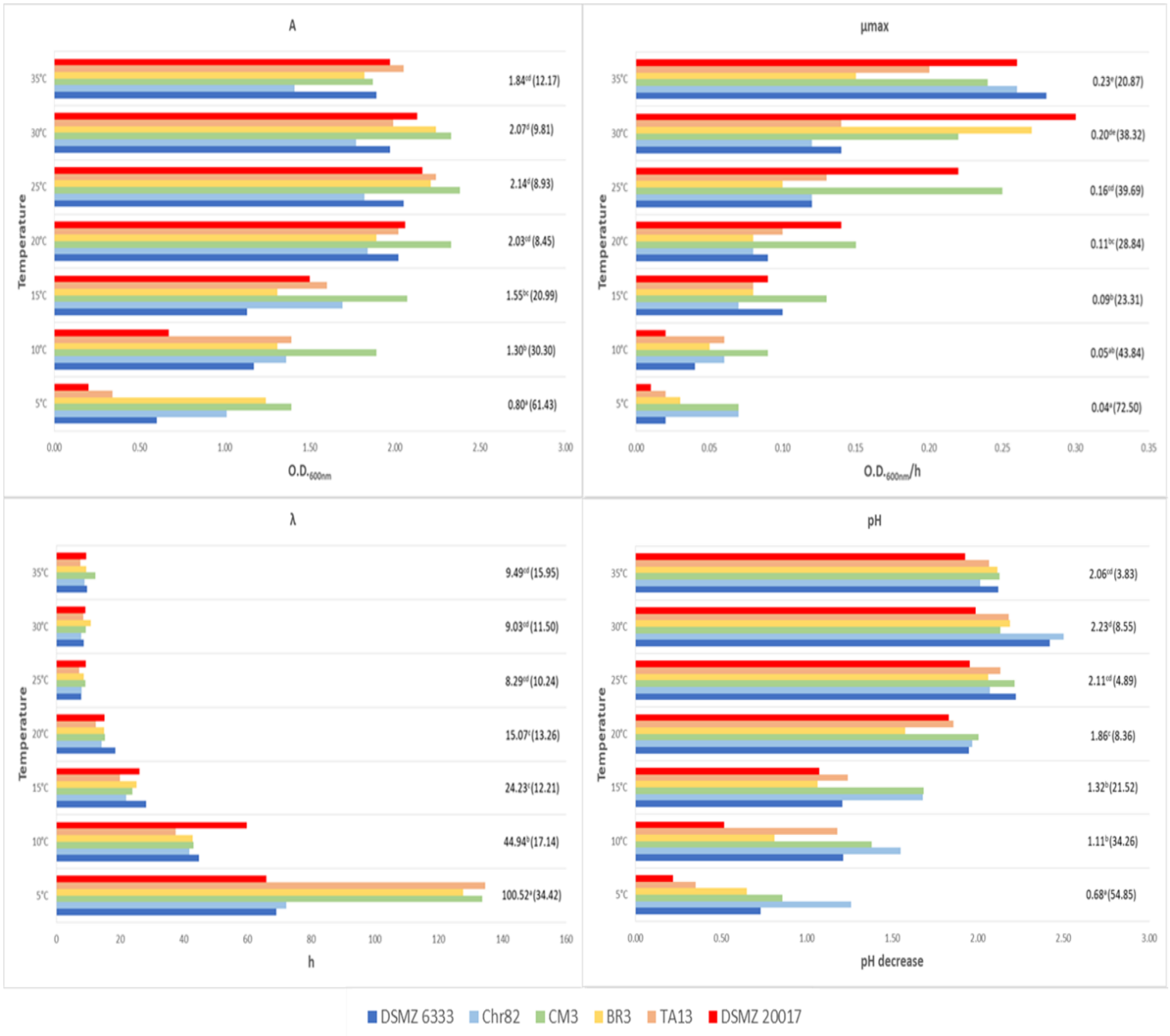

Figure 5.2.: Growth of *L. sakei* strains in MRS at different temperatures: estimates of the Gompertz parameters and pH decrease (initial value about 6.0). Are presents the maximum OD600 value reached, μmax is the maximum OD600 increase rate in exponential phase and λis the lag time.For each temperature, the mean value is reported and the relative coefficient of variation is indicated between brackets. Means with the same letter are not statistically different (P> 0.05) according to the post hoc (LSD test) comparison of ANOVA.

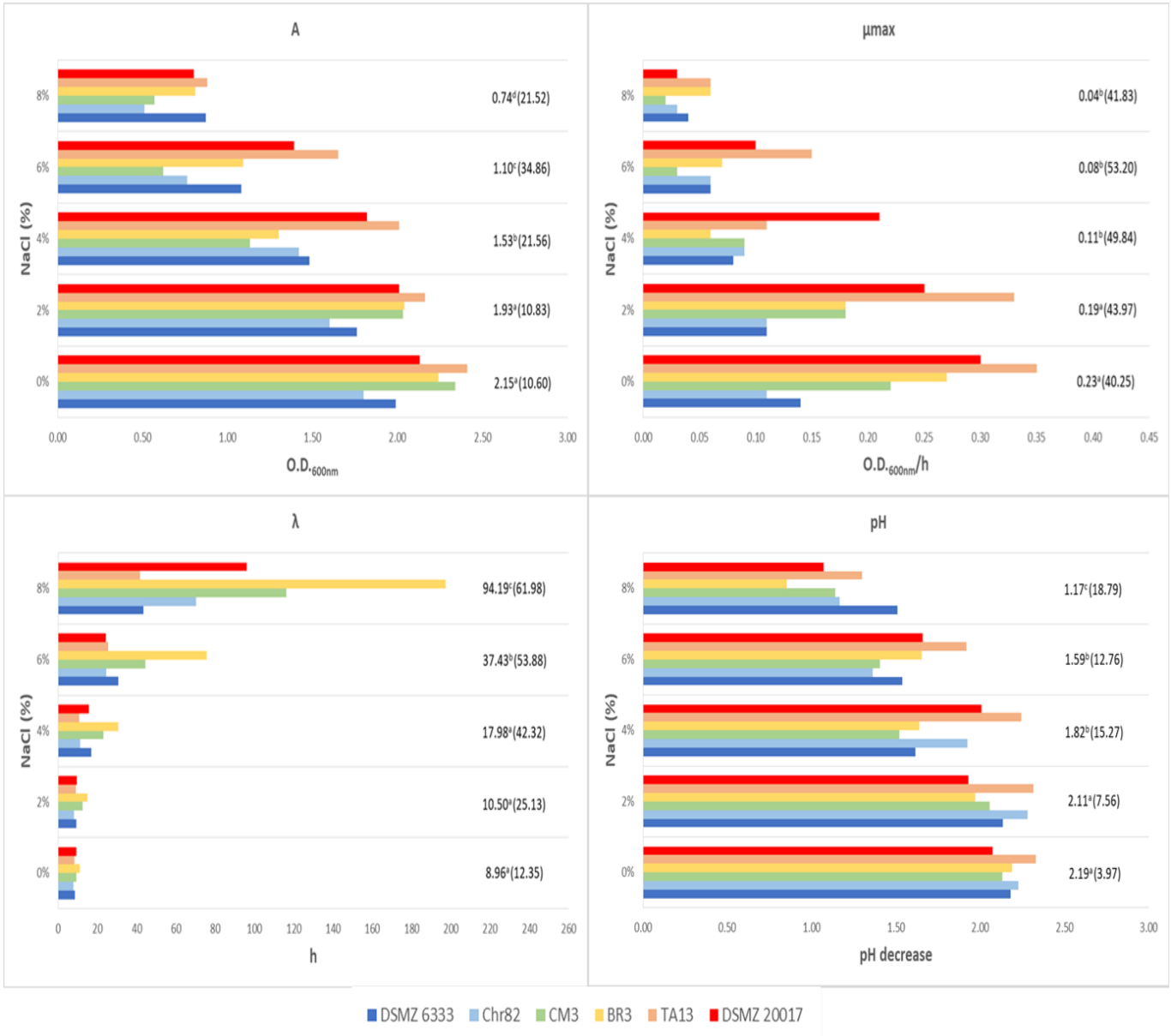

Figure 5.3.: Growth of *L. sakei* strains in MRS at different salt concentrations: estimates of the Gompertz parameters and pH decrease (initial value about 6.0). Are presents the maximum OD600 value reached, μmax is the maximum OD600 increase rate in exponential phase and λ is the lag time. For each NaCl concentration, the mean value is reported and the relative coefficient of variation is indicated between brackets. Means with the same letter are not statistically different (P> 0.05) according to the post hoc (LSD test) comparison of ANOVA.

5.3.2. SUSPENSION OF *L. SAKEI* RESTING CELLS IN DM

The suspension of the cells in DM was mainly aimed to evidence the physiological differences in relation to the pre-grown conditions and to the presence or the absence of sugars. The six *L. sakei* strains were pre-grown in MRS containing glucose or ribose as fermentable carbohydrates. According to the observations of McLeod et al. (2008), *L. sakei* strains grew less in the presence of ribose, and some, among which DSMZ 20017^t (used also in this trial), hardly grew. For this reason, small amounts of glucose (1 mM) were added in the medium containing ribose. Under these conditions, the transcription of ribose related genes can be initiated, and the pentose can be efficiently metabolized. The study of McLeod et al. (2011) demonstrated that *L. sakei* growth on ribose rather than glucose influenced not only the transcription of the genes strictly related to ribose catabolism, but also the transcription of several other genes responsible for alternative pathways was modified. In particular, many enzymes related to pyruvate metabolism were upregulated.

5.3.3. AMINO ACID METABOLISM

The content of the different amino acids was analyzed after 24 h of incubation. The results were expressed as percentage variation with respect to the initial concentration in the DM (Table 1) and are reported in Figure 5.4. as heat map. The results of serine and asparagine as well as histidine and glutamine are reported as sum of the percentage variation because the analytical method cannot satisfactorily separate these couples of amino acids. As it is possible to observe, many amino acids (such as glycine, proline, valine, isoleucine and leucine) presented limited changes with respect to their initial concentration. On the other hand, serine+asparagine, arginine, cysteine together with methionine were generally the more consumed amino acids, while only glutamate and, at a lesser extent, alanine, were characterized by a constant accumulation trend. According to a cluster analysis, it was possible subdivide the considered conditions in 6 clusters. The first grouped strains suspended in DM without sugar added, and namely BR3, TA13 (both 0-G and 0-R), Chr82 (0-G) and CM3 (0-R), which were mainly characterized by a relevant consumption of arginine. In the second cluster the consumption of arginine was accompanied by a relevant decrease of cysteine and serine+asparagine and characterized strain adapted to ribose such as Chr82 (both 0-R and 25-R), BR3 and CM3 (25-R). The strain DSMZ 20017^t suspended

in 25-R clustered alone and was characterized by high cysteine, aspartate and serine+aspragine consumption and glutamate, arginine, and alanine accumulation. The next cluster grouped three strain in 25-G (DSMZ 20017^t, DSMZ 6333 and BR3) which reduced the concentration of cysteine and serine+asparagine but not of arginine; moreover, these strains accumulated glutamate. The next group clustered DSMZ 20017^t and DSMZ 6333 suspended in DM without sugars and CM3 in DM 0-G and 25-G: in these conditions, the lower modifications of the amino acid profile of DM were observed. Finally, the last group (TA13 25-R and 25-G, Chr82 25-G and DSMZ 6333 25-R) were characterized by a relevant consumption of cysteine and by a reduction of serine+asparagine and an accumulation of glutamate.

The results concerning the amino acids characterized by the most relevant modification with respect to the initial concentration are reported in Figure 4.10. Moreover, the concentration (mM) of NH_3 and ornithine (absent in the not inoculated DM), detected with the same method used for amino acids, are reported in Figure 4.11.

The strains Chr82 and BR3 were efficient utilizers of arginine under all the condition tested. The strain TA13 preferably consumed this amino acid in the absence of sugars, while CM3 when pre-grown or incubated in the presence of ribose. The concentration of arginine was less affected by the strain DSMZ 6333, while surprisingly DSMZ 20017^t slightly increased its concentration. The ability to metabolize arginine through the arginine deiminase pathway (ADI), which brings to ATP production, is well known among LAB. Studies on *L. sakei* demonstrated that the species uses this pathway to improve its competitiveness and survival in meat, an environment relatively poor, for amount and diversity, in fermentable sugars but rich in arginine (Rimaux et al., 2012). Different efficiency in the utilization of arginine were also observed in two *L. sakei* strains (23K and LS25) grown in glucose limited medium; these differences were explained by the possible presence of two distinct ADI pathways, whose coexistence favored a more efficient arginine metabolism (McLeod et al., 2017).

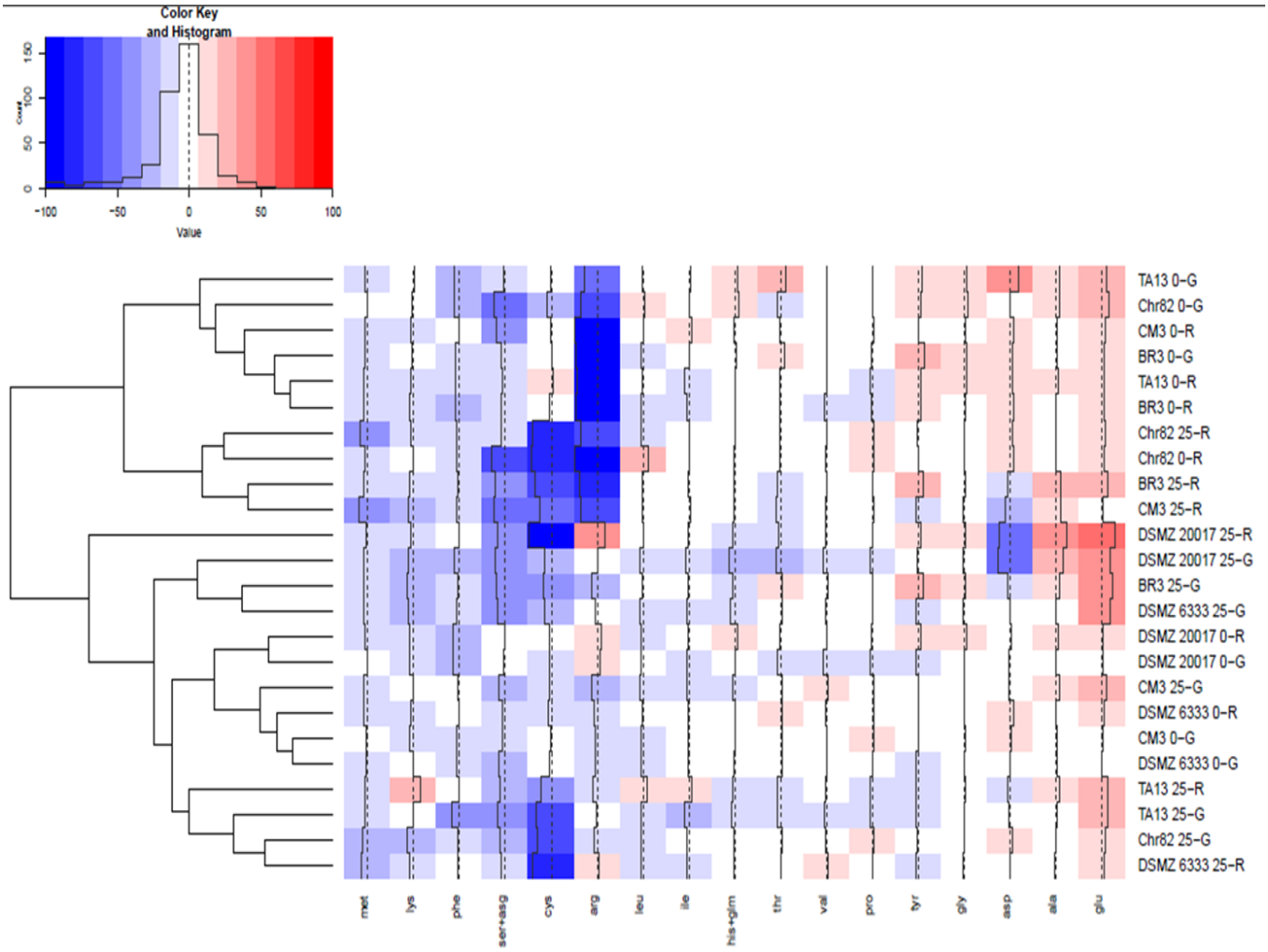

Figure 5.4.: Heat map relative to amino acid presence after 24 h of incubation. The quantitative change of each amino acid is expressed as percentage variation with respect to the initial concentration in DM (reported in Table 5.1). Increasing red intensity indicates increasing accumulation, whereas increasing blue intensity indicates increasing consumption. The dendrogram represents the hierarchical clustering process performed on the amino acid variations. A complete linkage approach on the Euclidean distance matrix was used.

According to previous study, the ADI pathway was inhibited by low pH (Rimaux et al, 2011a; Xu et al., 2015). This trend was confirmed by the fact that higher arginine depletion was observed when sugars were not added, and, thus, when the medium was not acidified by microbial metabolisms of simple carbohydrates. In particular, the highest level of arginine consumption was observed when the strains were pre-grown on ribose. Remarkable reductions of arginine concentration were also determined by some strains (Chr82, CM3 and BR3) suspended in DM containing ribose. McLeod et al. (2011) observed in the strain *L. sakei* 23K grown on ribose an up-regulation of the genes involved in the ADI pathway, but no modification in other two strains (LS25 and MF1053).

In addition to the generation of ATP, arginine conversion can result in the equimolar production of ornithine (Rimaux et al., 2011a). In effect, ornithine, which was not added in the DM, was accumulated in the media, and especially in those inoculated with cells pre-grown on ribose, independently of the presence of the pentose in DM (Figure 4.11). The results obtained are specular to arginine being its accumulation inversally proportional to arginine consumption. The strain DMSZ 20017^t, which did not consume arginine, did not produce ornithine.

The relation between arginine and ornithine has been confirmed in many conditions by the data reported here. Also the catabolic repression of ADI pathway by glucose (Fernández and Zúñiga, 2006) has been confirmed, given the generally lower utilization of arginine in the DM 25-G. In addition, a relevant variability in the possibility to metabolize this amino acid has been observed. One of the strain tested (DSMZ 6333) did not affect arginine content, while the strain DSMZ 20017^t accumulated this amino acid instead of metabolizing it, even in the absence of carbohydrates. This fact, in concomitance with the absence of ornithine, suggests that this strain lacks the ADI pathway. Ornithine was not detected in the sample Chr82 0-G (in which a high consumption of arginine was observed). Nevertheless, this was the only samples in which detectable amounts of putrescine (0.32 mM) were detected. In all the other strains was below the detection limit. Putrescine can be obtained by the direct decarboxylation of ornithine or through the agmatine deiminase pathways, whose presence have been described in *L. sakei* (Rimaux et al., 2012).

The concentration of serine+asparagine decreased in all the samples. The strains DMSZ 20017^t, BR3 and TA13 consumed these amino acids preferentially in the presence of sugars in DM (up to 20-40%), while the higher reductions were determined by Chr82 in the absence of sugars. Aspartate, which is one of two amino acid, together with glutamate, for which *L. sakei* is prototroph, was mainly accumulated, especially in the absence of sugars. By contrast, the strain DSMZ 20017^t in 25-G and 25-R was responsible for a remarkable consumption of this amino acid. Cysteine decreased in DM containing sugars, especially in 25-R, while Chr82 used a great amount of this amino acid in 0-R..

The consistent decrease of some amino acids during incubation of *L. sakei* strains indicated the possible existence of other metabolic routes aimed to produce energy. Serine utilization can play a relevant role in survival during stationary phase and its catabolism can be aimed

to increase the pool of pyruvate (Liu et al., 2003). The conversion of serine into pyruvate has been described in *P. pentosaceous* as the result of the activity of a serine dehydratase, without the intervention of transaminases, absent in *L. sakei* (Irmler et al., 2013). The degradation of serine with the consequent production of formate, succinate and acetate was described also in *L. plantarum* (Skeie et al., 2008). McLeod et al. (2017) observed a drastic utilization of serine and asparagine in *L. sakei* strains grown under glucose limiting conditions. Interestingly, they observed also an increase of the production of L-serine dehydratase.

Asparagine can be converted into aspartic acid with release of ammonia through the action of aspariginases, whose presence is documented among lactobacilli. Aspartate can further be catabolized through at least three pathways, two of which do not require the presence of a transaminase. Aspartate decarboxylase produces alanine and CO_2 and aspartase produces fumarate and ammonia. Both these enzyme activities have been found in LAB (Fernández and Zúñiga, 2006). Noteworthy, alanine can be converted in pyruvate by deamination and in these trials its concentration always increased (Figure 5.5.).

Also, cysteine can be metabolized to pyruvate, ammonia and hydrogen sulphide (Fernández and Zúñiga, 2006). The enzyme responsible for this pathway (cystathionine-γ-lyase) has been found in *L. fermentum* (Smacchi and Gobbetti, 1998), *Lc. lactis* (Fernández et al., 2002) and *L. reuterii* (Lo et al., 2009).

The availability of pyruvate, especially in the absence of sugars or in generally limiting nutritional conditions, can be the starting point for some pathways able to provide LAB with ATP or regenerate NAD with production of ethanol, acetate, diacetyl/acetoin (Gänzle, 2015). The generally higher activation of pathway that can supply pyruvate in the strains pre-grown or resuspended in ribose confirms the finding of McLeod et al. (2011) relative to the transcriptome of *L. sakei*. More recently, this possibility was discussed by McLeod et al. (2017) who support the hypothesis that, under glucose restriction, the pyruvate derived from amino acid metabolism can be the starting point for energy metabolic routes bringing to organic acid production. Nevertheless, in our study, a high variability in the physiological response among the strains was observed and univocal trends in relation of the sugar metabolized were not always found.

Figure 5.5.: Percentage variation with respect to the initial concentration in the DM of amino acids characterized by the most relevant modification after 24 h of incubation. The standard deviations are reported. For each strain, different letters mean significant differences in the amino acid variation in relation to the DM.

No relevant changes were observed in the concentrations of branched amino acids, whose metabolism is related to the production of aroma compounds important for the organoleptic profile of sausages. On the other hand, these metabolic pathways need the activity of aminotransferases that have never been signaled in *L. sakei* (Freiding et al., 2012).

Finally, the production of ammonia was higher for Chr82 in the samples without sugar added. On the contrary, DSMZ 20017^t produced ammonia only in the presence of sugars (Figure 5.6.). In any case, it was linearly related to the amino acid consumption (data not shown).

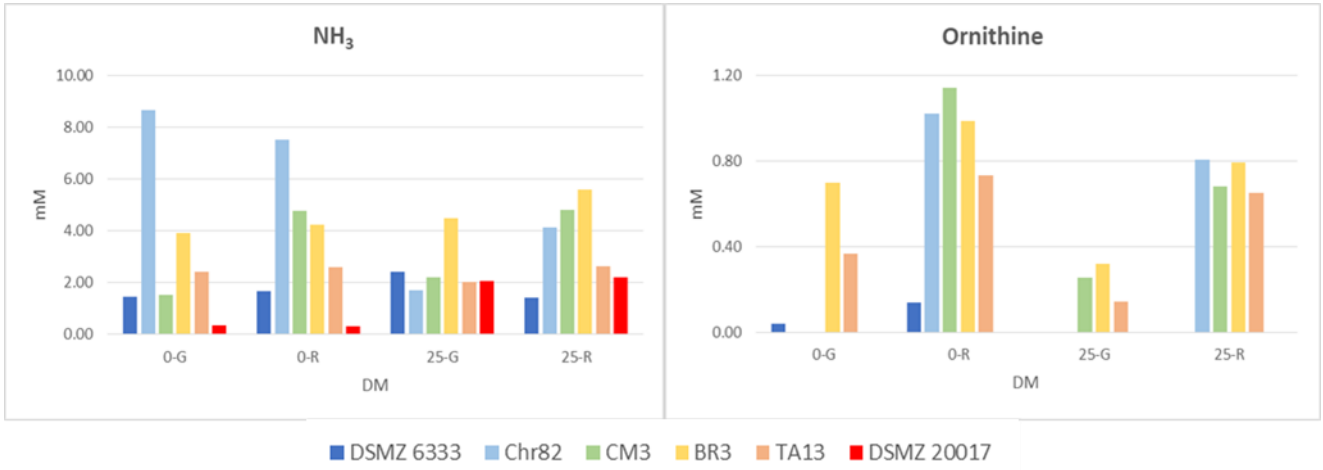

Figure 5.6.: Ornithine and NH_3 concentration in DM (expressed as mM) after 24 h of incubation. The standard deviations are reported. For each strain, different letters mean significant accumulation in relation to the DM.

5.3.4. ORGANIC ACID, DIACETYL AND ACETOIN PRODUCTION, PH AND CELL VIABILITY

The data reported in Table 5.2. show the production of D- and L-lactate, acetate and formate. As expected, when glucose or ribose were supplied, they were metabolized through the homofermentative and heterofermentative pathways, respectively. Glucose is mainly converted into L-lactate while D-lactate represent about 7% of the total lactic acid in the strain Chr82, while in the other strains it was always below 4%. On the contrary, D-lactate amount is higher in the fermentation of ribose (between 6.3% in strain CM3 and 26.3% in DSMZ 20017^t). The data reported by Malleret et al. (1998) demonstrated that *L. sakei* possesses only a L-lactate dehydrogenase (L-LDH) and a racemase able to partially convert it into D-lactate. In addition, given the specificity of the L-LDH, only L-lactate can be used for further metabolisms in nutritionally poor conditions. These findings were supported also by the work of McLeod et al. (2011). Different conclusions were observed for the strain *L. sakei* NRIC 1071 by Iino et al. (2003), who found two distinct dehydrogenases, a L-LDH and a D-LDH (the second acting later, in the stationary phase) while the presence of the

racemase was excluded. The data reported here for all the six strains studied showed the presence of a high L-lactate/D-lactate ratio confirming the presence of a specific L-LDH and, eventually, of a racemase.

In the DM containing ribose, the presence of lactate and acetate, as expected, was almost equimolar for most strains. The strains Chr82, TA13 and CM3 presented a prevalence of acetate. This prevalence has been observed in the primary metabolism of the bacterium (McLeod et al., 2010) and explained by the increased ATP production obtained through the phospoketolase pathway, but it can also be due to excesses of pyruvate deriving from other sources, such as amino acid metabolism (von Wright and Axelsson, 2011).

A great metabolic variability was observed in the amounts of fermentation products after 24 h among the tested strains. In the medium added with glucose, the concentration of lactate (D- and L-) varied from 21.31 (strain Chr82) to 41.13 mM (strain BR3). In the presence of glucose, also acetate was detected in amount higher than 2 mM in all the strains, except for CM3, suggesting the presence of other metabolic routes different from homolactic fermentation.

These considerations were confirmed also by the accumulation of organic acids (lactate and acetate) in the presence of 25 mM of ribose, which ranged from 33.44 mM (DSMZ 6333) and 52.06 mM (DSMZ 20017^{t}).

In the absence of sugars, the amounts of lactate is irrelevant (lower than 1 mM). Different results in the same conditions were observed for acetate, which was accumulated at higher concentration than lactate and reached concentration of 3.38 and 2.28 mM in Chr82 in 0-G and 0-R, respectively. Noteworthy, the higher acetate production in these conditions corresponded with the higher formate accumulation by the same strain (6.53 mM and 8.11 mM in 0-G in 0-R, respectively). High formate concentration (more than 1 mM) were also detected in the strain BR3 in the presence of sugars. The production of formate depends on the activation of pyruvate formate lyase (PFL) pathway, favored by anaerobiosis and substrate limitation (Gänzle, 2015), which allows the production of ATP from pyruvate. The incubation of the bacteria under ordinary atmosphere, seems to be in contradiction with the activation of this route. However, recently, it has been demonstrated that PFL pathway can be functional in aerobiosis when appropriate electron donors (ferredoxin, flavodoxin) are present (Zhang et al., 2015). Thus, it is possible that this way, when present, can be active in

L. sakei in the presence of a molecule such as cysteine, which lowers the redox potential acting as an electron donor (Shibata and Toraya, 2015). In any case, the production of acetate (in the samples not containing ribose) and formate indicates the possibility that excesses of pyruvate deriving from amino acids can be directed into pathways able to produce energy, such as pyruvate formate liase and pyruvate oxidase metabolisms (Gänzle, 2015). The dissipation of an excess of pyruvate leads also to the production of C4 aroma compounds, such as diacetyl and acetoin (Smid and Kleerebezem, 2014). Their production in these trials was determined by SPME and the results are reported in Table 2. The C4 compounds were never detected in the absence of sugars. In these conditions the excesses of pyruvate deriving from amino acid metabolism were used in energy producing pathways, as demonstrated by the accumulation of lactic and especially acetic acids. When sugars were added to the medium, the exceeding pyruvate was addressed to diacetyl/acetoin production, allowing NAD regeneration (von Wright and Axelsson, 2011). The presence of acetoin was always higher (with a ratio of about 2-3:1). The strains DSMZ 6333 and Chr82 presented the lower accumulation of C4 compounds, that were mainly produced in the presence of glucose. On the contrary, the remaining strains produced acetoin and diacetyl mainly in the presence of ribose. Interestingly, in the same analysis, ethanol was not detected or found only in traces (data not reported).

The decreases of pH reflected the organic acid accumulation and value between 2.94 (CM3) and 3.31 (TA13) were observed in the presence of glucose. The presence of ribose determined slightly lower decreases between 2.75 (Chr82) and 3.17 (BR3). In the absence of sugars, the pH did not significantly change.

Also, the viability of cells after 24 h of suspension in DM was monitored and the loss of viability was dramatically higher in the presence of sugars, partially attributable to the pH drop. The strains Chr82 and TA13 concentration decreased of about 2 log units and more in the presence of ribose or glucose, while the strains BR3 and CM3 were the more resistant (about one log decrease or less). In the absence of sugars added, the loss of viability was markedly lower (less than 1 log unit) except for the strain TA13.

Strain	DM[a]	Organic acids (mM)					Diacetyl[b]	Acetoin	Δ pH[c]	Δ log cfu/ml[d]
		L-lactate	D-lactate	Acetate	Formate	Lactate/ Acetate				
DSMZ 6333	0-G	0.12	0.14	0.65	0.32	0.41	0.0	0.0	0.14	-0.65
	0-R	0.10	0.09	0.41	0.05	0.48	0.0	0.0	0.19	-0.74
	25-G	29.99	0.66	3.64	0.00	13.83	9.7	18.3	-3.15	-1.69
	25-R	13.18	3.12	17.14	0.00	1.02	4.9	30.4	-2.85	-1.67
HJ-7	0-G	0.23	0.32	3.38	6.53	0.16	0.0	0.0	0.11	-0.31
	0-R	0.16	0.55	2.28	8.11	0.31	0.0	0.0	0.27	-0.47
	25-G	19.06	2.25	2.83	0.00	11.42	11.0	18.5	-2.82	-2.52
	25-R	15.60	3.26	21.67	0.00	0.92	5.0	13.3	-2.75	-1.60
CM3	0-G	0.23	0.10	0.70	0.01	0.47	0.0	0.0	0.21	-0.62
	0-R	0.08	0.05	1.44	0.14	0.09	0.0	0.0	0.31	-0.18
	25-G	22.18	0.54	1.37	0.14	24.73	6.3	11.2	-2.94	-1.00
	25-R	16.63	1.39	25.63	0.00	0.72	16.7	54.0	-2.80	-0.96
BR3	0-G	0.10	0.20	0.39	0.15	0.76	0.0	0.0	0.20	-0.02
	0-R	0.18	0.22	0.35	0.14	1.12	0.0	0.0	0.32	0.09
	25-G	39.69	1.44	2.42	1.64	17.00	3.9	9.8	-3.30	-0.75
	25-R	19.03	2.93	25.53	1.02	0.99	13.6	64.1	-3.17	-0.94
TA13	0-G	0.19	0.10	0.20	0.05	1.43	0.0	0.0	-0.04	-1.46
	0-R	0.19	0.10	0.36	0.06	0.83	0.0	0.0	0.12	-1.60
	25-G	36.38	1.72	2.55	0.13	19.16	4.8	12.3	-3.31	-2.35
	25-R	18.95	1.78	24.21	0.75	0.87	16.6	51.6	-2.99	-1.96
DSMZ 20017[t]	0-G	0.23	0.06	0.17	0.25	1.73	0.0	0.0	-0.11	0.01
	0-R	0.22	0.05	0.17	0.07	1.52	0.0	0.0	-0.10	-0.10
	25-G	30.14	0.20	2.96	0.31	10.25	9.3	18.8	-3.12	-0.69
	25-R	19.15	7.27	25.64	0.62	1.04	32.7	89.0	-2.97	-0.51

Table 5.2.: Organic acid, diacetyl and acetoin accumulated by the six *L. sakei* strains in the defined media after 24 h of incubation. Also the pH variation and the decrease of cell viability are reported.

[a]Defined medium. [b]Data are expressed as ratio between peak area of each molecule and peak area of the internal standard (4-methyl-2-pentanol). [c]Difference between initial pH and pH after 24 h of incubation at 30°C. [d]Difference between initial cell concentration and cell concentration after 24 h of incubation at 30°C. [e]Not detected. [f]Under the detection limit (0.01 mM).

5.4. CONCLUSIONS

The metabolic heterogeneity observed among the six *L. sakei* strains confirmed the metabolic differences already observed within this species, also in relation to the genomic sequences described. All the strains could grow between 5 and 35°C (but not at 40°C) and in the presence of salt concentration up to 8%, but with growth parameters characterized by an extreme variability. In addition, the pre-grown conditions, and in particular the sugar added, induced the activation/repression of different pathways resulting in different phenotypes. The use of a defined medium allowed the study of amino acid metabolism. As

expected, the metabolism of arginine was particularly active, even if it was not observed, under the adopted conditions, in the type strain DSMZ 20017^{t}. However, other amino acids (serine+asparagine, cysteine, methionine) were metabolized, after deamination, through potentially energetic pathways starting from pyruvate accumulation. This was demonstrated by the organic acid accumulation (especially acetate) in the DM without sugar added and also by the acetate accumulation in the presence of glucose. In addition, the presence of excesses of pyruvate deriving from amino acids lead to the accumulation of diacetyl and acetoin by all the strains when sugars were added, where the energetic needs of the cells could be satisfied by primary metabolism. These results are an important starting point to better understand the metabolisms of this species and to implement this knowledge from a productive point of view. In fact, the exploitation of the relevant phenotypic biodiversity of *L. sakei* can be a key factor for optimizing the performance of starter cultures used in meat-fermented foods.

CHAPTER 6

METABOLISM OF *LACTOBACILLUS SAKEI* CHR82 IN THE PRESENCE OF DIFFERENT AMOUNTS OF FERMENTABLE SUGARS

6.1. INTRODUCTION

Lactobacillus sakei is species showing a high level of adaptation for meat environments in which it can rapidly grow and efficiently compete with other species present as component of the microbial communities of this raw material. Because of this aptitude, selected strains of this species are widely used as starter cultures in meat fermentation for dry sausages production (Cocconcelli and Fontana, 2010). The technological and safety advantage of the use of this species as starter culture consists in the ability to inhibit pathogenic as well spoilage microorganisms, to grow at low temperature and the ability to colonize the habitat during all the ripening (Chaillou et al., 2013). This latter aspect, essential for guaranteeing the quality of fermented sausages throughout all the steps of production and commercialization, depends on its ability to efficiently produce metabolic energy even when the hexoses, which are fermented through the homofermentative pathway, are completely depleted. In fact, this species can also ferment the pentoses contained in the nucleosides via phospoketolase pathway, as demonstrated by McLeod et al. (2008) and Rimaux et al. (2011b).

Moreover, the arginine deiminase (ADI) pathway is active, even if with different efficiency, in *L. sakei* and is an important additional energy sources giving a competitive advantage in matrices with low fermentable sugar concentration but rich in this amino acid, such as meat (McLeod et al., 2017; Montanari et al., 2018b).

The mean genotype size of *L. sakei* is relatively small (approx. 2020 kb) and reflects this specialization even if a great variation was observed within the species (about 25%) (Chaillou et al., 2009). The adaptation to growth in proteinaceous matrices (meat and fish) explains the absence of genes responsible for amino acid anabolism, particularly transaminases: *L. sakei* strains are auxotrophic for all the amino acids, with the exception of aspartate and glutamate (Chaillou et al., 2005). Nevertheless, the metabolisms of some on these compounds are crucial for explaining the success of this species in the colonization of fermented meat. In addition to the use of arginine as energy supply, other amino acids may be useful for the strategies of this bacterium. Serine may be deaminated by L-serine dehydratase yielding a surplus of pyruvate and relevant uptakes of this molecule in defined media by *L. sakei* have been observed (McLeod et al, 2017; Montanari et al., 2018b). Also, threonine (McLeod et al, 2017) and cysteine (Montanari et al., 2018b) were depleted in

remarkable amounts (higher than those required by the generation of the intracellular amino acid pool) by this species under defined conditions. Also, the presence of a gene coding for L-threonine dehydrogenase active in some *L. sakei* strains has been described; this protein catalyses the conversion to glycine via 2-amino-3-ketobutyrate with a concomitant NADH+ reduction (McLeod et al, 2017).

Survival and growth in environments poor in fermentable sugars have been also explained by an efficient pyruvate metabolism carried out for generating further ATP and gaining reducing power (regeneration of NADH+). The pyruvate formate lyase (PFL) pathway lead to the possible accumulation of by-products such as formate, acetate and ethanol in anaerobic or reducing conditions while, in aerobic condition CO_2 and acetate may be produced through the pyruvate oxidase (POX) pathway and the pyruvate dehydrogenase complex (PDC). Enzymes involved in these pathways were found in *L. sakei* and their transcription was enhanced in the presence of pentoses as fermentable substrate (McLeod et al, 2010; McLeod et al., 2011**).**

The same Authors (McLeod et al., 2017) also demonstrated that glucose availability can affect different parameters such as growth rate, fermentative pathway (i.e. shift from homolactic towards more mixed acid fermentation), amino acid consumption and gene expression, but no effect on cell viability (in terms of percentage of alive cells) was observed. They hypothesised that this condition of low glucose availability is for *L. sakei* analogous to the so-called “complete caloric restriction”, that in eukaryotes, from single-celled yeast to humans, is a conserved mechanism that results in expanded healthy life span in response to a reduction of energy intake.

A previous work was addressed to the evaluation of the metabolic response of resting cells of six strains of *L. sakei* in relation to the sugar present and its amount (Montanari et al., 2018b).In this work, the metabolic activity of one of this strains (Chr60) was studied after its growth in a medium containing sugars at different concentrations, focusing the attention on its metabolome, that is the complete set of small metabolites it consumed or produced (Fiehn, 2002). This was done on the assumption that the metabolome would be the best representation of the microorganism’s phenotype, being downstream of the genome, transcriptome, and proteome.

The analytical platform selected for the purpose was ^{1}H-NMR, whose ease of sample preparation and high reproducibility was expected to counterbalance the low sensitivity (Laghi, et al., 2014). Previous works convinced us about the suitability of this approach; for example, Biagioli et al. (2017) were able to observe divergent metabolic activities of two batches of the same probiotic preparation. Parolin et al. (2015) identified the metabolome traits distinguishing vaginal lactobacilli with different anti-candida activity. Picone et al. (2013) followed the adaptation of *Escherichia coli* 555 to increasing doses of carvacrol.

In this work, the strains *L. sakei* Chr82, already tested by Montanari et al. (2018b), has been chosen to evaluate its different metabolic profile following the growth in a defined medium (DM) in relation to the sugar added (glucose or ribose) and to the their initial concentrations: 25 mM (optimal growth condition), or 2.5 mM (limiting growth conditions), in order to simulate a harsh environment in which sugars are limited. The strain was chosen because it is used as commercial starter cultures in fermented sausage production.

Moreover, conversely to the previous trials (Montanari et al., 2018b) in which cells were inoculated at very high concentrations (about 9 log CFU/ml) and incubated for 24 h (allowing the reaching of stationary phase) to assess the consumption of sugar and amino acids and the resulting accumulation of organic acids and other metabolites, in this trial the cells were inoculated at about 7 log CFU/ml in order to monitor also growth performances in the different conditions.

The different cell suspensions were monitored for 48 h by measuring OD_{600nm} and pH evolution and after 24 and 48 hours of incubation samples were collected to analyse:

- Cell cultivability (by plate counting)
- Organic acid accumulation and amino acid consumption (by HPLC)
- Metabolome analyses (by ^{1}H-NMR)
- Viability and cell membrane permeability and depolarization (by flow cytometry).

In this way the resulting phenotypic activity was therefore measured with two different approaches: the first was a chromatographic approach based on HPLC analyses, while the second was focused on NMR investigation of the metabolome characterizing the different conditions. The results obtained with the two approaches were compared and discussed.

Moreover, the use of flow cytometry at this stage of the research was helpful to better investigate the physiological state of *L. sakei* cells in different growth conditions and

therefore to clarify the mechanisms behind the ability of this species to well survive in fermented meats for long period. From a technological point of view, this could improve knowledge to optimize the use of this starter culture in food industry.

6.2. MATERIALS AND METHODS

6.2.1. MICROORGANISM USED

The commercial strain *Lactobacillus sakei* Chr82, supplied by the company Chr. Hansen (Parma, Italy), used as a starter culture in the production of fermented cured meats, was used.

6.2.2. GROWTH MEDIA

6.2.2.1. MODIFIED MRS

For the pre-cultivation of *L. sakei* Chr82 a modified MRS medium was used with the addition of two different sugars: in one case a quantity equal to 4.5 g / L of glucose was added, while in the other were 3.75 g / L of ribose was added.

In the case of MRS supplemented with ribose, according to the observation by McLeod et al. (2008), a small amount of glucose was also added (0.2 g / L) in order to stimulate the growth of the microorganism in the initial phase.

6.2.2.2. DEFINED MEDIUM

After the pre-cultivation in modified MRS, the cells were collected and suspended again in a defined medium (DM), whose composition is shown in Table 6.1.

The media obtained was added with two different carbon sources, namely glucose and ribose, at different concentrations: 25 mM or 2.5 mM which contains small amounts of glucose. From growth experiments in DML when 0.02% glucose was added in addition to 0.5% ribose, values similar to those obtained from DMLG was observed (results not shown). The small amount of glucose added clearly provides the growth needed for ribose-related genes to be initiated and for ribose to be efficiently metabolized by the bacterium.

Compound	Concentration (g/L)	Compound	Concentration (g/L)
Sodium acetate	2.0	L-Alanine	0.2
K_2HPO_4	1.75	L-Arginine	0.2
$MnSO_4\ 4H_2O$	0.012	L-Aspartic acid	0.2
$MgSO_4\ 7H_2O$	0.2	L-Asparagine	0.2
Tween 80	1 mL	L-Tryptophan	0.2
		L-Serine	0.2
Thiamine HCl	0.0011	L-Phenyalanine	0.2
Folic acid	0.0002	L-Histidine	0.2
Riboflavin	0.001	L-Isoleucine	0.2
Calcium panthotenate	0.001	L-Leucine	0.2
Nicotinic acid	0.001	L-Lysine	0.2
p – Aminobenzoic acid	0.0004	L-Methionine	0.2
Piridoxal	0.0005	L-Proline	0.2
		L-Threonine	0.2
Adenine	0.005	L-Valine	0.2
Guanine	0.01	L-Tyrosine	0.2
Uracil	0.01	L-Cysteine	0.2
		L-Glutamine	0.2
		L-Glutamic acid	0.2
		Glycine	0.2

Table 6.1.: Defined medium composition

6.2.3. EXPERIMENTAL PLAN

L. sakei Chr82 was pre-grown in modified MRS (with glucose or ribose) and incubated overnight at 30°C. The cells grown in modified MRS were collected by centrifugation at 10.000 rpm for 10 minutes. The cells of *L. sakei* grown in the presence of glucose were suspended again in DM with 2.5 mM of glucose and subsequently inoculated in the two DM added with glucose 2.5 mM (2.5 G) or 25 mM (25 G), at a cell concentration of about 7 log

CFU/mL. The same procedure was applied for *L. sakei* Chr82 cells grown with ribose, but in this case the cells were suspended again in DM with ribose 2.5 mM and the inoculated (cell load 7 log CFU/ml) in the two DM added with ribose 2.5 mM (2.5 R) or 25 mM (25 R).

The obtained samples were incubated at 30°C and monitored at different times: T_0 (initial incubation time), T_{24} (after 24 hours) and T_{48} (after 48 hours).

Growth performances were analysed by measuring the increase of the optical density at 600 nm (OD_{600nm}) using a Spectrophotometer Jenway, 6705 UV- Vis.

6.2.4. MICROBIOLOGICAL ANALYSIS

The microbiological counts of *L.sakei* Chr82 was carried out at each time defined by the experimental plan by taking 1 mL of the sample, diluted in sterile physiological water to obtain a first dilution 1:10, from which the subsequent decimal dilutions were prepared and plated by surface spatulation on MRS Agar (Oxoid, Basingstoke, UK). The plates were then incubated at 30°C for 48 hours.

6.2.5. ORGANIC ACIDS CONTENT

The quantification of organic acids was performed using a HPLC instrument (intelligent HPLC PU-2089 quaternary pump, UV 2070 Plus multi-length UV-VIS detector, Jasco Corp., Tokyo, Japan) equipped with a Rheodyne manual injector with loop from 20µl (Rheodyne, Rohnert Park, CA, United States), while the column is HP-87H Bio-Rad with a size of 300 mm x 7.8 mm (Bio-Rad Laboratories, Hertfordshire, United Kingdom). For the analysis, which took place under isocratic conditions, the sample was centrifuged and subsequently filtered with 0.22 µm porosity filters to remove the cells. The sample was then injected into the instrument. As a mobile phase, H_2SO_4 was used at a concentration of 0.005 M and a flow of 0.6 mL / min was applied with a constant temperature at 65°C. A UV-type detector set at a wavelength of 210 nm was used. Chromatographic peaks were identified by comparing retention times with those of standards (Sigma-Aldrich) and quantification was carried out by using the external standard method.

6.2.6. QUANTIFICATION OF AMINO ACIDS

To evaluate the variation of amino acid concentration in the various growth conditions, samples were subjected to a derivatization through the use of the AccQ-Fluor Reagent kit (Waters Corp., Milford, MA, United States), following the manufacturer's protocol. First, the AQC reagent (6-aminoquinolyl-N-hydroxysuccinimide carbamate) was reconstituted in acetonitrile (Waters Corp.) at a final concentration of 10 mM. Subsequently, 10 μL of sample were derivatized with 70 μL of AccQ-Fluor Borate Buffer (Waters Corp.) and 20 μL of reconstituted reagent. The samples were then heated at 55 ° C for 10 minutes. The derivatized sample was analyzed by HPLC (PU-1580 Intelligent HPLC, Intelligent Fluorescence Detector FP-1520 and Intelligent Sampler AS-2055 Plus, with 10 μl loop, Jasco Corp.), using the AccQ-TagTM column (3.9 mm × 150 mm). The analysis was carried out at a constant temperature of 30°C by a counter-gradient run at a speed of 1 mL / min. The two mobile phases used are:

- Solution consisting of 100 mL of Eluent AccQ-Tag (Waters Corp.), diluted 1:10 with H_2O by chromatography (Sigma-Aldrich, St. Louis, MO, United States);
- Solution composed of 60% acetonitrile and 40% H_2O by chromatography (Sigma-Aldrich, St. Louis, MO, United States).

The fluorescent detector was set at the excitation wavelength of 250 nm and an emission wavelength of 395 nm.

6.2.7. FLOW CYTOMETRIC ANALYSIS

Flow cytometry is a technique created in the 1970s for the analysis of blood cells but only used since the 1990s in other areas, such as the food industry, and allows cells that are suspended in a liquid medium to be measured and characterized. This system consists of a fluidic system in which the cell suspension is inserted. The cells arriving at the measurement point are hit by a focused light beam coming from the laser. Signals are then generated following the encounter between the light beam and each individual cell present in the fluid, and these are collected by a system of lenses, mirrors and optical filters and then sent to the respective sensors that allow the measurement of the intensity. Finally, each electrical impulse generated by each sensor is sent to a data analyzer, which then allows the graphic representation and processing. The instrument used is the Accuri C6 flow cytometer

(BD Biosciences, Milan, Italy) with the following parameters set: FSC threshold 5000, SSC threshold 4000, total events collected 30000, medium flow rate. All the parameters were acquired on a logarithmic scale and the data obtained were analyzed with the BD software ACCURITM C6 version 1.0 (BD Biosciences). Before the analysis, the sample taken was diluted (where necessary) in the respective buffer up to a concentration of 7 log CFU/mL, optimal cell density for a correct marking of the sample by fluorochromes.

For each condition, 4 aliquots (100 µl for each one) were taken and marked differently with specific dyes:

- an aliquot was analyzed without any dye (unstained sample);
- an aliquot was stained with 1 µl of SYBR green I (Sigma-Aldrich), a fluorochrome that evaluates the relative fluorescence in the green channel (filter 533/30) and allows to distinguish the cells from the background (particles). In this case, the fluorochrome binds to cellular DNA, whether the cell is alive, dead or in a phase of latency;
- an aliquot was stained with 3 µl DiBAC4 (3) 3 µM, a marker used to evaluate the membrane potential, as it is able to enter the cell and confer fluorescence if the cell membrane is depolarized;
- an aliquot was stained with 2 µl of a solution of SYBR green I 1X and propidium iodide (PI, Sigma-Aldrich) 0.5 µg / ml 1:1. This dual staining allows to discriminate three sub-populations: viable cells, damaged cells, dead cells.

Before analysis, each aliquot was kept at 37°C for 15 minutes in order to let the dye to react with the cells.

6.2.8. DETERMINATION OF OPTICAL DENSITY AND pH MEASUREMENT

The measurement of the optical density of the samples for the determination of the microbial load and for the construction of growth curve was carried out at a wavelength of 600 nm (OD_{600}), through a UV-VIS spectrophotometer (Jenway, 6705 UV- Vis). Before each detection, a calibration of the instrument was performed with the blank (non-inoculated medium) of the respective sample. The pH meter Basic 20 (Crison Instrument, Barcelona, Spain) was used for pH measurement.

6.2.9. DATA MODELING WITH THE GOMPERTZ EQUATION

The results of the optical density were modeled with the STATISTICA program (Statsoft Italia, Vigonza, Italy) through the Gompertz equation (Zwietering et al., 1990):

$$y = k + Ae - e\,[(\mu max \times eA)\,(\lambda - t) + 1]$$

where y is the OD_{600} at time *t*, *A* represents the maximum OD_{600} value reached, μ_{max} is the maximum OD_{600} increase rate in exponential phase and λ is the lag time.

6.2.10. METABOLOMICS ANALYSIS BY ^{1}H-NMR

For metabolomics investigation by ^{1}H-NMR, an analysis solution was created, with 3-(trimethylsilyl)-propionic-2,2,3,3-d4 acid sodium salt (TSP) 10 mM in D_2O, set at pH 7.00±0.02 by means of 1M phosphate buffer. The solution contained also 10 µL of NaN_3 2 mM, to avoid microbial proliferation, while TSP was employed as NMR chemical-shift reference, as suggested by Zhu et al. (2019). Growth medium samples were prepared for ^{1}H-NMR by thawing and centrifuging 1 mL of each for 15 min at 18630 g and 4°C. 700 µL of supernatant were added to 200 µL of NMR analysis solution. Finally, each of the so obtained samples were centrifuged again at the above conditions right before analysis.

^{1}H-NMR spectra were recorded at 298 K with an AVANCE III spectrometer (Bruker, Milan, Italy) operating at a frequency of 600.13 MHz, equipped with the software Topspin 3.5. Following the procedure described by Laghi et al. (2014), The HOD residual signal was suppressed by applying the first increment of the nuclear Overhauser effect spectroscopy (NOESY) pulse sequence and a spoil gradient. This was done by employing the NOESYGPPR1D sequence, part of the standard pulse sequence library. Each spectrum was acquired by summing up 256 transients using 32 K data points over a 7184 Hz spectral window, with an acquisition time of 2.28s, and spectra were elaborated with R package (R Core Team, 2018) as reported by Zhu et al. (2019).

6.3. RESULTS

6.3.1. DETERMINATION OF GROWTH CURVES AND MICROBIOLOGICAL ANALYSIS

The DM containing the two different sugars (glucose and ribose) at the two different concentrations (2.5 and 25 mM) were inoculated with approx. 7 log CFU/ml of *L. sakei* Chr82. The growth dynamics monitored by measuring the OD_{600} are reported in Figure 6.1. The experimental data were modelled with the Gompertz equation (Zwietering et al., 1990). The amount of sugars influenced the maximum OD_{600} which reached values of 0.264 and 0.282 in the presence of glucose and ribose at 2.5 mM, respectively, while, under the same conditions the maximum OD_{600} predicted when the sugars were added at 25 mM were 1.446 and 1.151. The addition of ribose determined slightly shorter λ time and lower μ_{max}.

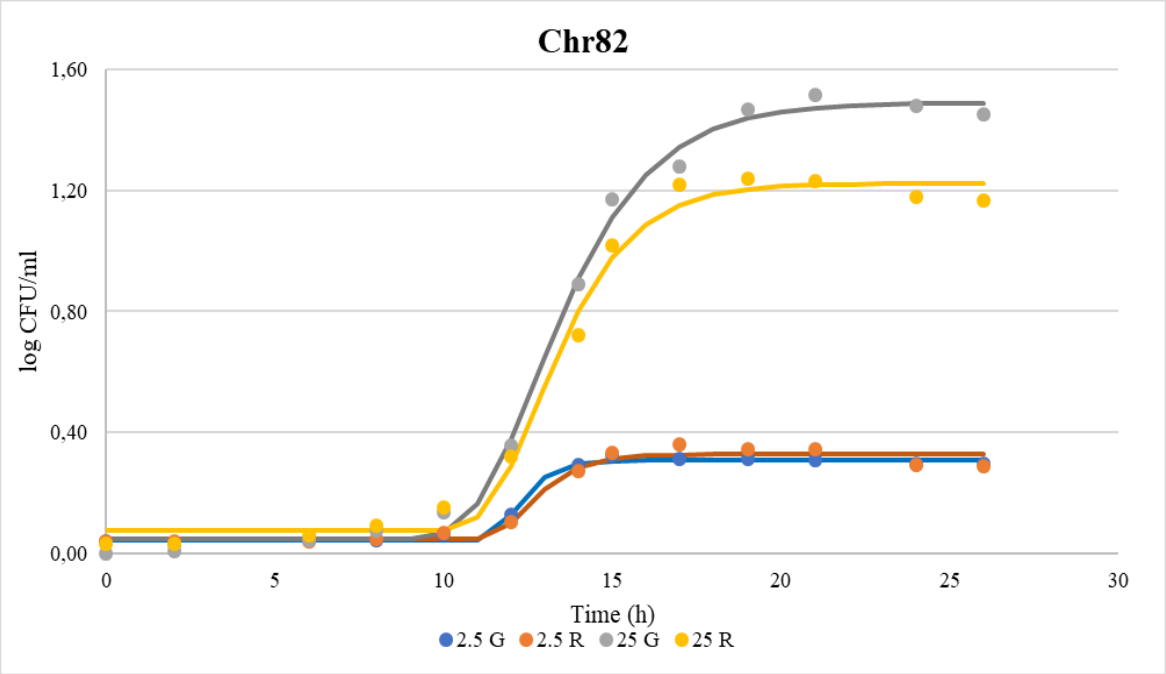

Figure 6.1.: Growth curves of *L. sakei* Chr82 at 30°C under different conditions.

The final cell concentrations were determined also by plate counting and are reported in Figure 6.2. No significant *L. sakei* cell counts were found after 24 h. As observed, after 48 cell counts showed a drastic decline (1 log unit or more) if compared with the counts at 24 h (Figure 6.1) and the higher survival rate was observed in the sample containing 2.5 R. The rapid beginning of the death phase in culture media for this species has already been

observed (Fadda et al., 2010) and it is in contrast with the long survival showed by the same cells in more strictly conditions as those characterizing fermented sausages during ripening.

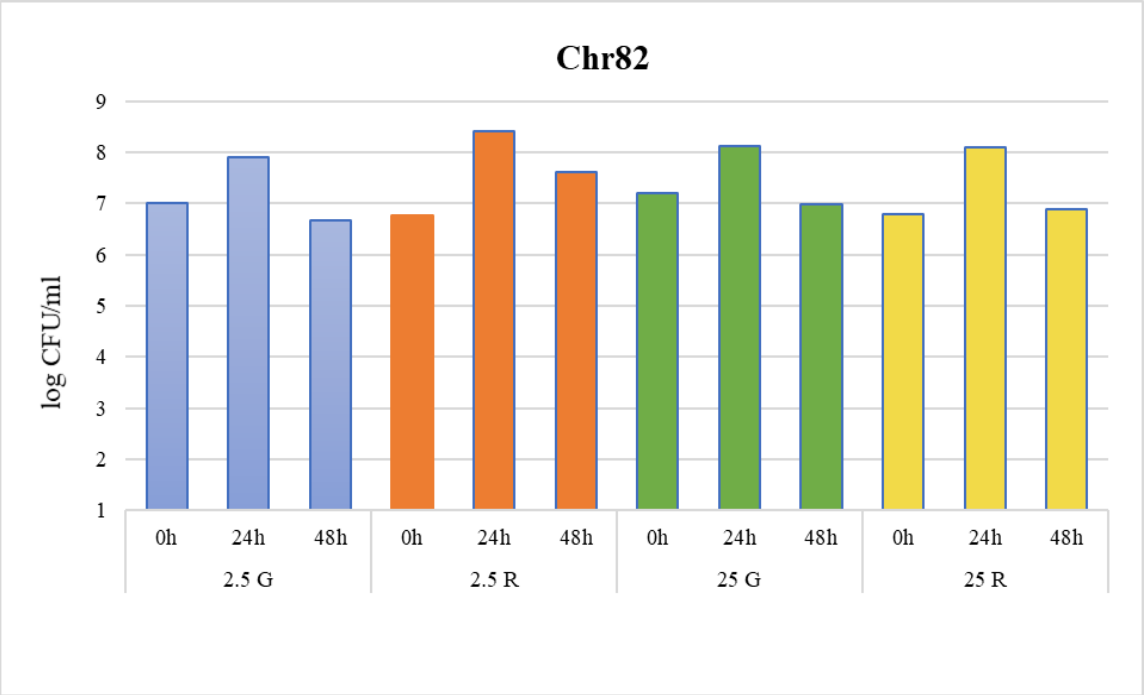

Figure 6.2.: Cellular load expressed in log CFU/mL following the incubation of *L. sakei* Chr82 at 30 ° C under different conditions.

6.3.2. ORGANIC ACID CONTENT AND pH

In Table 6.2 the organic acids accumulated after 24 and 48 h of incubation are reported.

Sample		Time	L-lactic acid (mM)	D-lactic acid (mM)	Acetic acid (mM)	Δ Acetic acid	pH values
Control			0.23	-	23.72	-	6.50
Chr82	2.5 G	24h	4.25	-	24.24	0.52	5.78
		48h	3.88	-	24.33	0.61	5.83
	2.5 R	24h	1.66	-	26.57	2.85	6.17
		48h	1.39	-	26.77	3.15	6.21
	25 G	24h	39.60	0.41	26.30	2.58	4.15
		48h	43.81	0.39	26.50	2.77	4.00
	25 R	24h	15.61	1.43	44.72	21.00	4.73
		48h	16.02	1.09	46.43	22.70	4.37

Table 6.2.: Organic acids content and pH values of *L. sakei* Chr82 samples incubated at 30 ° C under different conditions

Considering the lower sugar concentration (2.5 mM) their conversion into metabolic compounds was always higher than 90% expressed as mM of the acid in relation to the sugars. Only L-lactate was detected in these samples. In the presence of glucose, its presence (about 4 mM) was accompanied by lower proportion of acetate (0.52 and 0.61 mM after 24 and 48 h, respectively). More acetate was produced, as expected, in the presence of ribose. In addition, the molar production of the two acids represented more that 90% of the theoretical yield. In the presence of ribose, the ratio acetate/lactate was higher than 1, indicating the activation of pathways alternative to homolactic and heterolactic fermentations (Axelsson, 2004).

The presence of fermentable carbohydrates at 25 mM determined the production of more than 40 mM of lactate and small amounts (2 mM) of acetate in the medium added with glucose while the addition of ribose resulted in the accumulation of lactate (more than 17 mM, including L- and D-lactate) and relevant quantities of acetate (more than 21 mM). In this latter case, the quantitative production of acetate was higher than expected (acetate/lactate molar ratio higher than 1) as a consequences of the activity of secondary pathways. As observed by McLeod et al. (2010) *L. sakei* alters its pyruvate metabolism, when grown in the presence of ribose) generating more ATP per ribose unit up-regulating pyruvate decarboxylases and pyruvate dehydrogenases.

The presence of glucose compared to ribose always determined a lower pH (Table 6.2) and after 48 h values of 5.83 vs. 6.21 were found with the addition of the sugars at 2.5 mM while at 25 mM the pH measured were 4.00 and 4.37.

6.3.3. AMINO ACID QUANTIFICATION

It is well known that *L. sakei*, as a consequence of its adaptation to meat environments, is auxotrophic for 18 amin oacids (Chaillou et al., 2005). The study of the variations of amino acid content in a defined medium is important to elucidate how the species uses these molecules. In first instance, they are used to assembly all the proteins (and enzyme) necessary to sustain growth and multiplication. However, it is interesting to evidence alternative uses of the amino acids in the perspective of explaining the high persistence of *L. sakei* cells in habitats, such as fermented sausage, in which fermentable sugars are rapidly depleted. In a previous work Montanari et al. (2018) described amino acid variations due to

the resuspension of resting cells in a define medium. Here, the variation induced in the amino acid content in a defined medium was analysed because of the growth of *L. sakei* in the media described above.

		Asp ac	Ser+Asp	Glu ac	Gly	His+Glu	Arg	Thr	Ala	Pro	Cys	Tyr	Val	Met	Lys	Ile	Leu	Phe
Initial value (mM)		1.62	3.92	1.62	2.68	2.78	1.28	1.64	2.15	1.74	1.09	0.82	1.58	1.24	1.14	1.40	1.53	1.27
Sample	**Time**																	
2.5 G	**24h**	1.50	3.61	1.51	2.93	2.36	1.12	1.75	2.12	1.93	1.44	0.83	1.71	1.17	1.14	1.38	1.40	1.34
		(-7.49)	(-7.94)	(-6.53)	(9.53)	(-15.27)	(-12.00)	(6.57)	(-1.09)	(10.49)	(32.02)	(0.85)	(7.68)	(-5.45)	(-0.45)	(-1.54)	(-8.51)	(6.02)
	48h	1.37	3.04	1.27	2.52	1.93	0.32	1.78	1.95	1.80	1.62	0.78	1.54	0.63	1.20	1.41	1.49	1.48
		(-15.53)	(-22.41)	(-21.36)	(-5.97)	(-30.44)	(-74.57)	(7.29)	(-9.22)	(3.20)	(48.56)	(-5.83)	(-2.90)	(-48.88)	(4.96)	(0.68)	(-2.22)	(16.51)
2.5 R	**24h**	1.42	3.41	1.40	2.83	2.23	0.11	1.61	2.12	1.78	1.33	0.82	1.57	1.25	1.13	1.45	1.44	1.32
		(-12.26)	(-13.00)	(-13.50)	(5.61)	(-19-94)	(-91.57)	(-1.87)	(-1.38)	(2.09)	(21.78)	(0.12)	(-0.96)	(1.18)	(-0.67)	(3.63)	(-6.03)	(4.53)
	48h	1.44	2.97	1.29	2.49	1.87	0.21	1.68	1.93	1.77	1.67	0.86	1.50	0.87	1.18	1.41	1.50	1.40
		(-11.04)	(-24.42)	(-20.47)	(-6.82)	(-32.85)	(-83.76)	(2.08)	(-10.03)	(1.75)	(53.09)	(3.93)	(-5.05)	(-30.22)	(2.99)	(0.39)	(-1.58)	(10.72)
25 G	**24h**	1.56	1.38	2.07	2.72	1.52	1.00	1.53	1.85	1.69	1.16	0.80	1.47	1.21	1.03	1.47	1.40	1.49
		(-4.00)	(-64.72)	(27.56)	(1.78)	(-45.33)	(-21.38)	(-6.93)	(-14.09)	(-3.07)	(6.31)	(-2.87)	(-7.27)	(-2.42)	(-9.96)	(4.93)	(-8.25)	(17.71)
	48h	1.41	1.02	1.94	2.34	1.15	0.88	1.33	1.66	1.76	1.22	0.77	1.39	1.16	0.92	1.28	1.46	1.39
		(-13.27)	(-73.95)	(19.88)	(-12.43)	(-58.54)	(-30.78)	(-19.13)	(-22.80)	(0.81)	(21.05)	(-7.05)	(-12.17)	(-6.27)	(-19.61)	(-8.81)	(-4.67)	(9.38)
25 R	**24h**	1.39	2.35	1.58	2.64	1.88	0.10	1.74	1.94	1.75	0.95	0.76	1.47	1.06	1.01	1.37	1.46	1.42
		(-14.55)	(-40.17)	(-2.17)	(-1.41)	(-32.31)	(-92.08)	(5.53)	(-9.78)	(0.30)	(-13.05)	(-7.88)	(-6.93)	(-14.50)	(-11.82)	(-2.04)	(-4.22)	(11.82)
	48h	1.30	2.10	1.40	2.37	1.57	0.14	1.61	1.78	1.81	1.04	0.80	1.41	0.96	0.91	1.24	1.38	1.39
		(-20.06)	(-46.41)	(-13.32)	(-11.60)	(-43.72)	(-89.31)	(-2.14)	(-17.08)	(3.60)	(-5.09)	(-2.27)	(-11.15)	(-22.76)	(-20.45)	(-11.72)	(-9.93)	(9.45)

Table 6.3.: Amino acid content detected by HPLC analysis in DM after 24 and 48 hours of incubation of *L. sakei* Chr82 at 30 ° C under different conditions. The data are reported concentration of each amino acid (mM) in the samples, while the percent variation with respect to the initial amount in the medium is reported in brackets.

In Table 6.3 the influence of the growth on the amino acid content is expressed as percent decrease of the content of each amino acid expressed as mM (see Table 6.1 for the initial concentration) as revealed by HPLC analyses.

The concentration of many amino acids after 24 and 48 h of incubation showed small variations with respect to the initial level.

Aspartate, alanine, valine, lysine and leucine were always consumed in amount lower than 20% of the initial concentration. Smaller variations were observed for isoleucine, tyrosine, threonine, and glycine. Phenylalanine was accumulated (up to 17% of the initial concentration) in all the conditions tested with a trend similar to that observed by Mc Leod et al. (2017). Glutamic acid, generally consumed in all the other conditions, was accumulated in the presence of glucose 25 mM. The remaining amino acids were subjected to relevant variations.

The use of arginine to produce ATP through the ADI pathway has been well studied in *L. sakei* (Rimaux et al., 2012). However, different patterns of decrease have been observed among strains (Montanari et al., 2018; McLeod et al., 2017). In this case, the presence of glucose retarded its depletion even if after 48 h the presence 2.5 mM of this sugar caused a high consumption of this amino acid (Figure 6.3). The activation of the ADI pathway is considered crucial for permitting the survival of this species in meat environment. Some strains possess a second putative ADI pathway which improves their ability to take advantages from the high amounts of arginine in meats (McLeod et al., 2017).

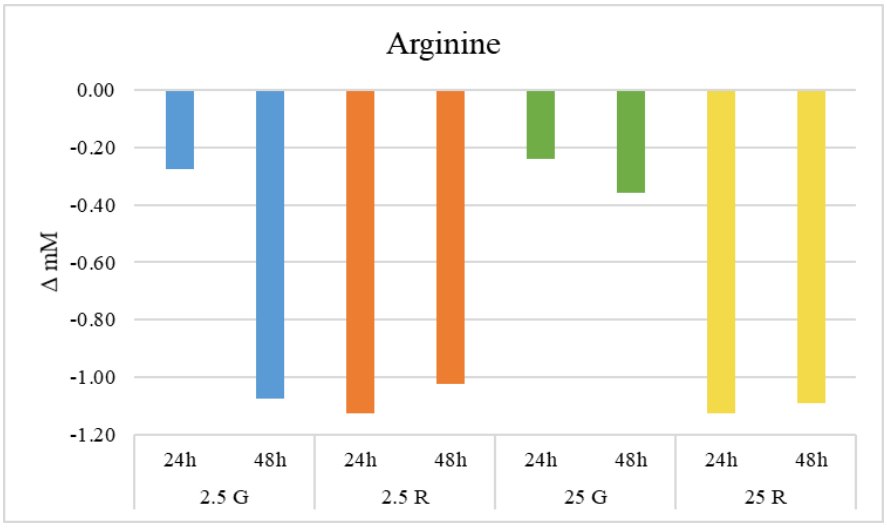

Figure 6.3.: Variation of the amount of arginine present in the DM after incubation for 24 and 48 at 30 ° C of the Chr82 strain of *L. sakei* under different conditions

This trend is confirmed by the accumulation of ornithine (Figure 6.4). Ornithine is the final product of ADI pathway and was produced in higher amount in the presence of ribose, particularly 2.5 mM. The absence of a correlation between arginine consumption and ornithine production can be attributed to the ability of this strain to decarboxylate this amino acid. In fact, only *L. sakei* Chr62, among the six strains tested by Montanari et al. (2018), was able to produce putrescine from the decarboxylation of ornithine.

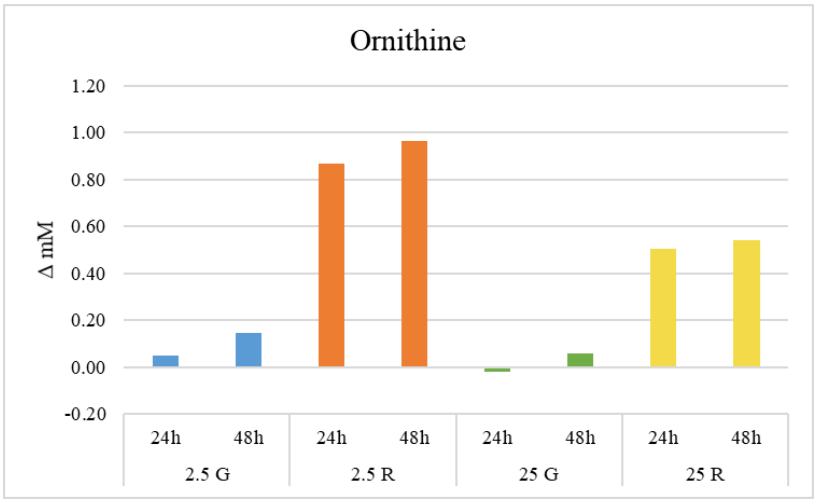

Figure 6.4.: Production of ornithine after incubation at 24 and 48 hours at 30°C of the *L. sakei* Chr82 under different conditions

The sum of serine and asparagine (not separated under the adopted HPLC analytical protocol) showed a drastic decrease, especially in the samples containing high sugar concentration and after 48 h of incubation (Figure 6.5). Serine can be used to supply pyruvate, which can then be used to produce energy through the PFL or POX (Ganzle 2015). The conversion of serine into pyruvate has been described in *P. pentosaceus* as the result of the activity of a serine dehydratase (Irmler et al., 2013) while *L. plantarum* can metabolize serine with the production of formate, succinate and acetate (Skeie et al., 2008). McLeod et al. (2017) showed a high use of serine and asparagine in *L. sakei* strains grown under glucose limiting conditions. Among the six *L. sakei* strains tested by Montanari et al. (2018), under resting conditions, the strain Chr82 was the most efficient in serine+aspargine uptake in the absence of fermentable sugar. The decrease was higher in the sample containing glucose, in contrast to the trend observed for arginine.

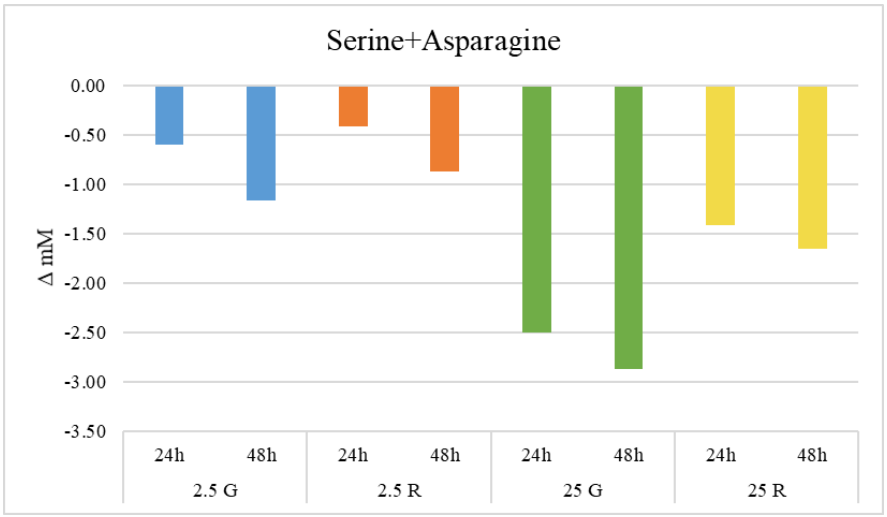

Figure 6.5.: Variation in the amounts of serine and asparagine present in the DM after incubation for 24 and 48 hours at 30°C of *L. sakei* Chr82 under different conditions.

The decrease observed for histidine+glutamine (not separated under the adopted HPLC analytical protocol) was higher again in the media containing glucose (Figure 6.6). The resuspension of resting cells of the same strain in defined medium did not markedly change the concentration of these amino acids. By contrast, growing cell decreased the concentration of these amino acids, especially after 48 h and when glucose was present to the medium.

McLeod et al. (2017) showed a strong decrease of glutamine during a continuous cultivation in a glucose-limited medium inoculated with two *L. sakei* strains, while the concentration of histidine was scarcely affected.

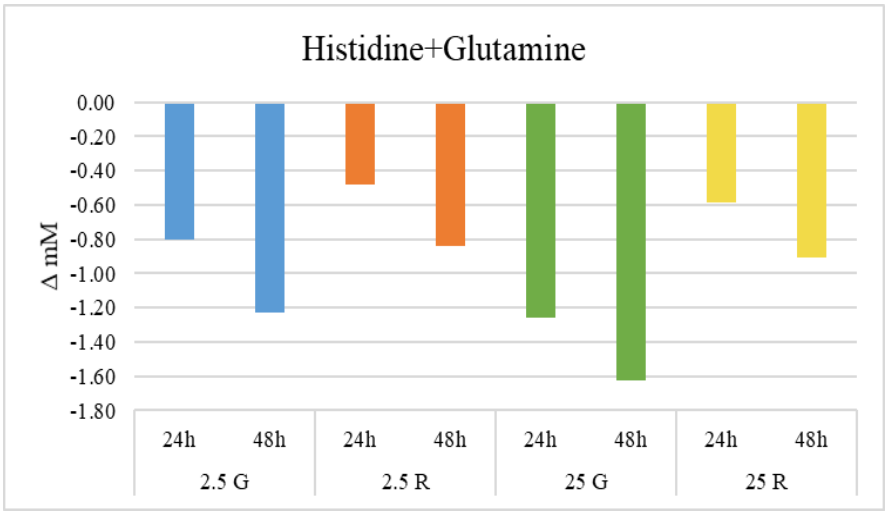

Figure 6.6.: Variation of the amounts of histidine and glutamine present in the DM after incubation for 24 and 48 hours at 30°C of *L. sakei* Chr82 under different conditions.

Finally, the sulfur amino acids methionine and cysteine showed a correlated trend (Figures 6.7 and 6.8). Relevant diminutions of methionine were observed after 48 h in the samples with 2.5 mM of both sugars. However, these decreases were accompanied by concomitant increases of cysteine. Only the samples containing ribose 25 mM presented a simultaneous decrease of both amino acids.

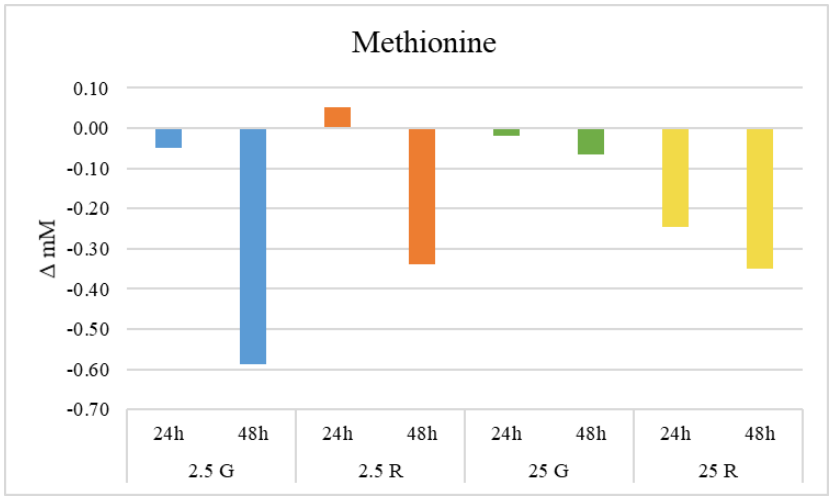

Figure 6.7.: Variation in the amounts of methionine present in the DM after incubation for 24 and 48 hours at 30°C of *L. sakei* Chr82 under different conditions.

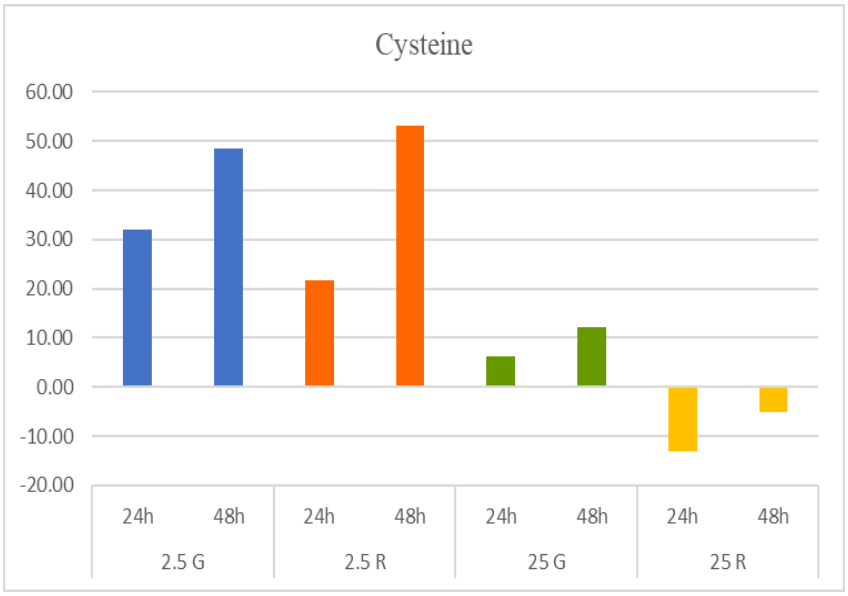

Figure 6.8.: Accumulation or consumption of cysteine in the DM after incubation for 24 and 48 hours at 30°C of *L. sakei* Chr82 under different conditions.

6.3.4. FLOW CYTOMETRIC ANALYSIS

The same samples were also subjected to flow cytometric analysis to define some parameters linked to cell viability. Each sample was labeled with SYBR green I and propidium iodide (PI) in a 1:1 ratio. This dual staining allowed to discriminate three sub-populations: viable cells, damaged cells, dead cells. The results are shown in Figure 6.9.

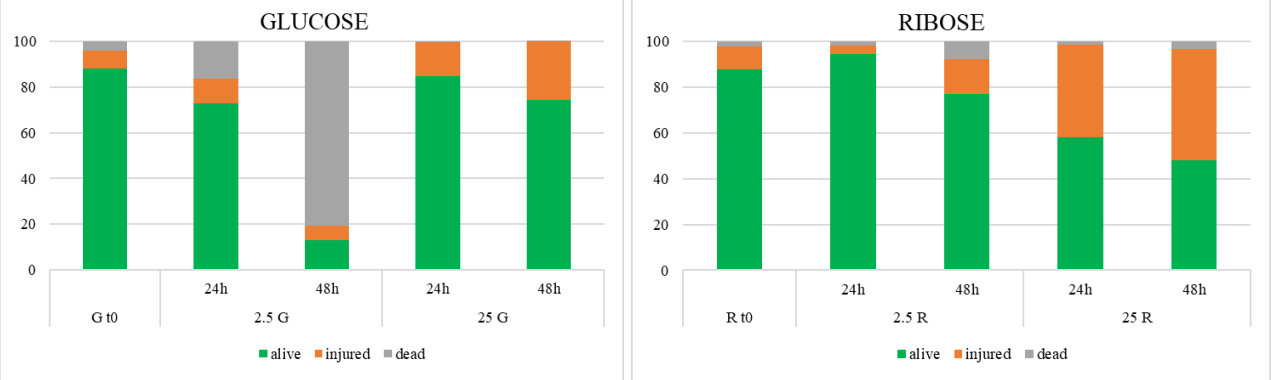

Figure 6.9.: Distribution of live, damaged and dead cells of the *Lactobacillus sakei* Chr82 strain after incubation at 24 and 48 hours at 30°C in different conditions. The data are reported as the relative frequency of the total population obtained by cytofluorimetric analysis with double marking (SYBR-Green I and PI).

As far as the samples grown on glucose, a higher viability in the cells grown in the presence of 25 mM of this sugar was generally observed. The percentage of cells recognized as alive was 84.6% and 74.4%, after 24 and 48 hours, respectively. At the same time the quantity of dead cells passed from 14.9% to 79.0% and injured cells from 0.5% to 7.2%. The presence of limited amount of glucose (2.5 mM) determined a drastic increase of the dead cells (approx. 18% after 24 h and 80% after 48 h). When the ribose was added at the higher concentration (25 R), the number of dead cells remained comparable to those observed in the presence of glucose, but the portion of damaged cells was much more relevant, already starting from 24 hours of incubation. Conversely, the addition of ribose at 2.5 mM resulted, after 48 hours, in a higher viability if compared to the sample added with the same amount of glucose.

Regarding the membrane depolarization, expressed as fluoresce of DiBAC4(3), the results obtained for the two sugars at the two concentrations (Figure 6.10) showed a degree of depolarization which is inversely proportional to the pH. In fact, in the 25 G and 25 R samples the low pH values (4 in the presence of glucose and 4.6 in the presence of ribose)

determined a greater depolarization in the cell membrane. In the 2.5 G and 2.5 R samples, characterized by higher pH values (about 5.8 in the presence of glucose and 6.2 in the presence of ribose), the depolarization degree is lower.

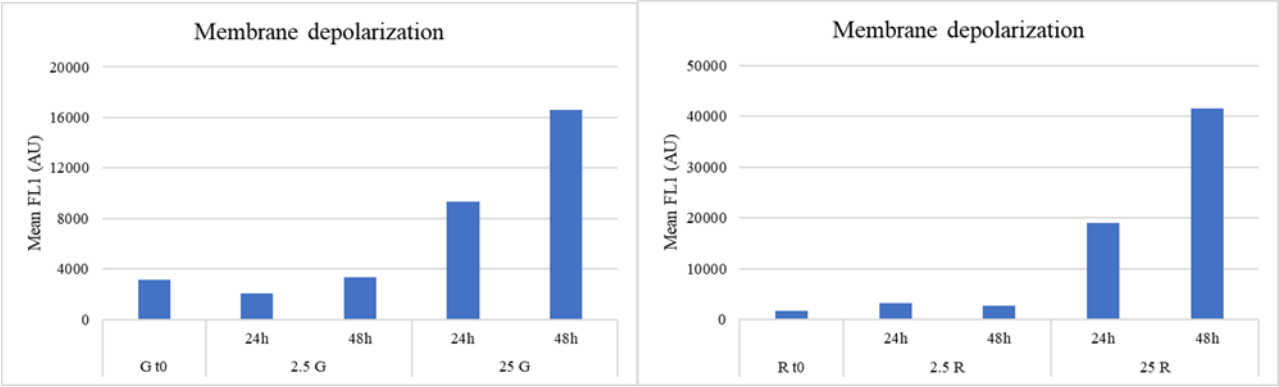

Figure 6.10: Cytofluorimetric analysis related to membrane depolarization in samples incubated with glucose or ribose at different concentrations. Data are reported as mean fluorescence of the DiBAC4 (3) dye (arbitrary unit, AU).

Finally, the results of membrane permeability (Figure 6.11) showed higher values in the presence of 2.5 mM of glucose, while the trend was opposite in the presence of ribose, where the membrane permeability was higher in the presence of the higher concentration of this sugar.

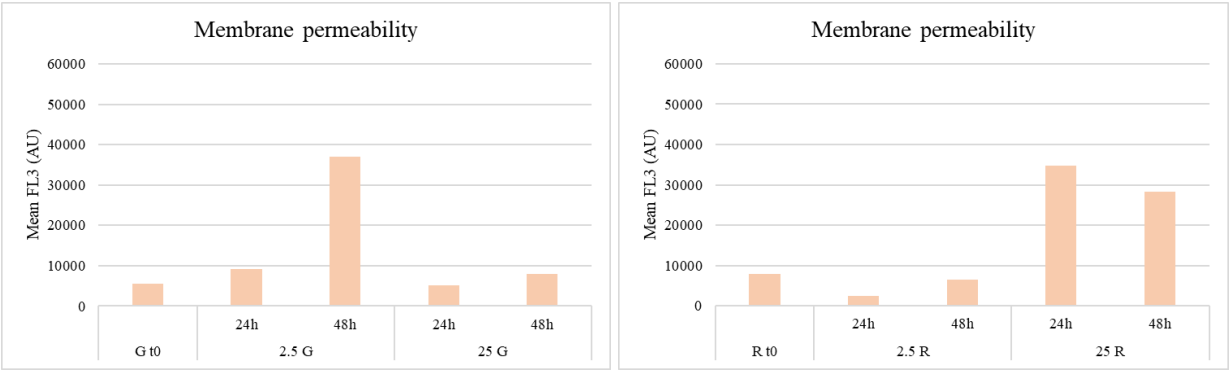

Figure 6.11: Cytofluorimetric analysis related to membrane permeability in samples incubated with glucose or ribose at different concentrations. Data are reported as mean fluorescence of the PI (arbitrary unit, AU).

6.3.5. METABOLOME ANALYSIS BY ^{1}H-NMR

With the aim to have a deeper insight on the metabolomic responses of the strain *L. sakei* Chr82, the same samples were further analysed applying an NMR protocol. Regarding the amino acid concentration, the correlation between NMR and HPLC results was satisfying, as demonstrated by the regression analysis reported in Figure 6.14, characterized by a high R^2 (0.8702), an intercept of 0.1542 and an angular coefficient close to 1 (1.1372).

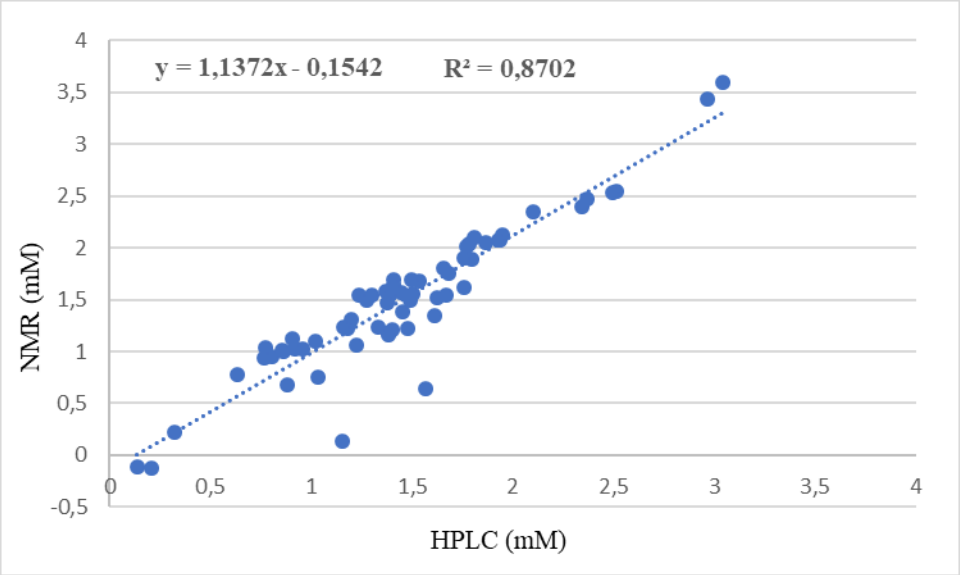

Figure 6.13: Correlation matrix between NMR and HPLC results (expressed as mM)

These data were focused, in first instance, to the evaluation of the amino acids which were not separated by HPLC analysis, i.e. serine+asparagine and histidine+glutamine. The results relative to these amino acids, expressed as percentage variation, are reported in Figures 6.12 and 6.13.

Taking into consideration serine+asparagine, the total decrease of these two molecules detected with the two methods were comparable. However, the NMR approach indicated that the major diminution concerned serine (Figure 6.12), confirming the hypothesis that this amino acid can provide a supply of pyruvate, which can be addressed to alternative metabolic pathways important when available sugars become a limiting factor. Liu et al. (2003) proved the central role of pyruvate deriving from serine in L. plantarum metabolism, demonstrating that it was involved in the regeneration of NADH+ and in the production of ATP, acetate, formate, ethanol, acetoin, diacetyl and 2-3-butanediol.

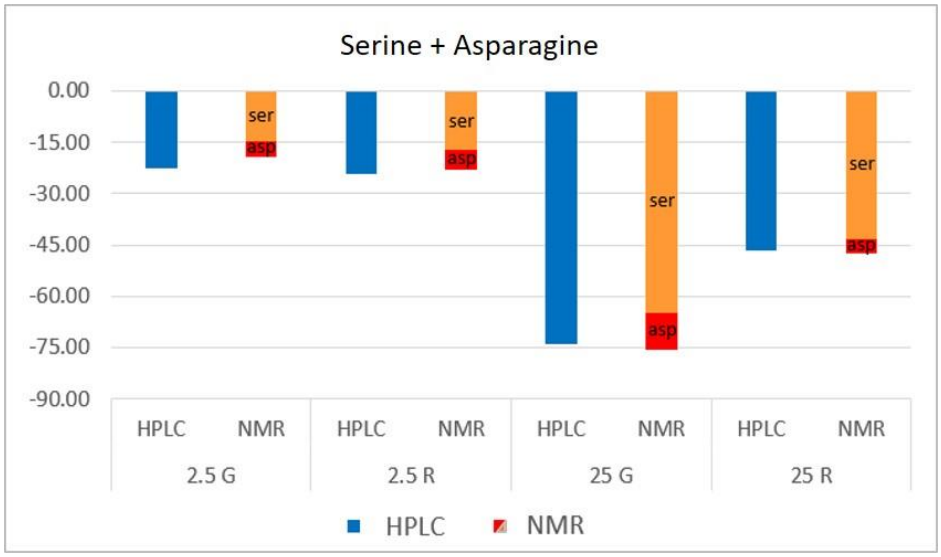

Figure 6.12: Percentage variation with respect to the initial concentration in the DM of serine and asparagine in *L. sakei* Chr82 after 48 hours of incubation at 30°C in different conditions.

Lower concordance was observed for the data of histidine+glutamine (Figure 6.13). While in the presence of low sugar concentration the NMR analysis reported lower amounts of amino acid uptake if compared to HPLC results, an opposite trend characterized the samples with the higher glucose and ribose concentration. In any case, according to the NMR results, the consumption of the two amino acids was equivalent.

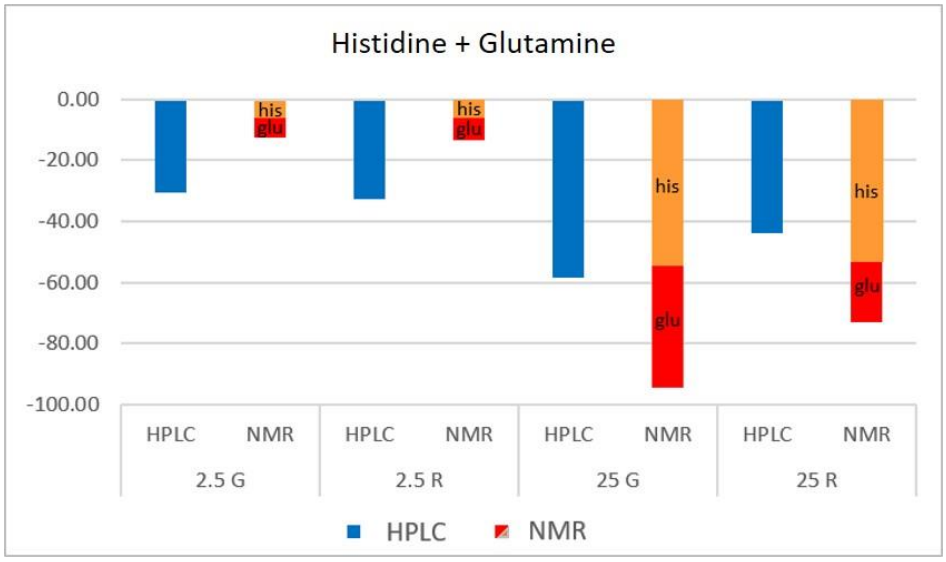

Figure 6.13: Percentage variation with respect to the initial concentration in the DM of histidine and glutamine in *L. sakei* Chr82 after 48 hours of incubation at 30°C in different conditions.

A PCA was carried out by using the percentage mM variations of amino acids with respect to the initial concentration. The results are reported in Figure 6.14.

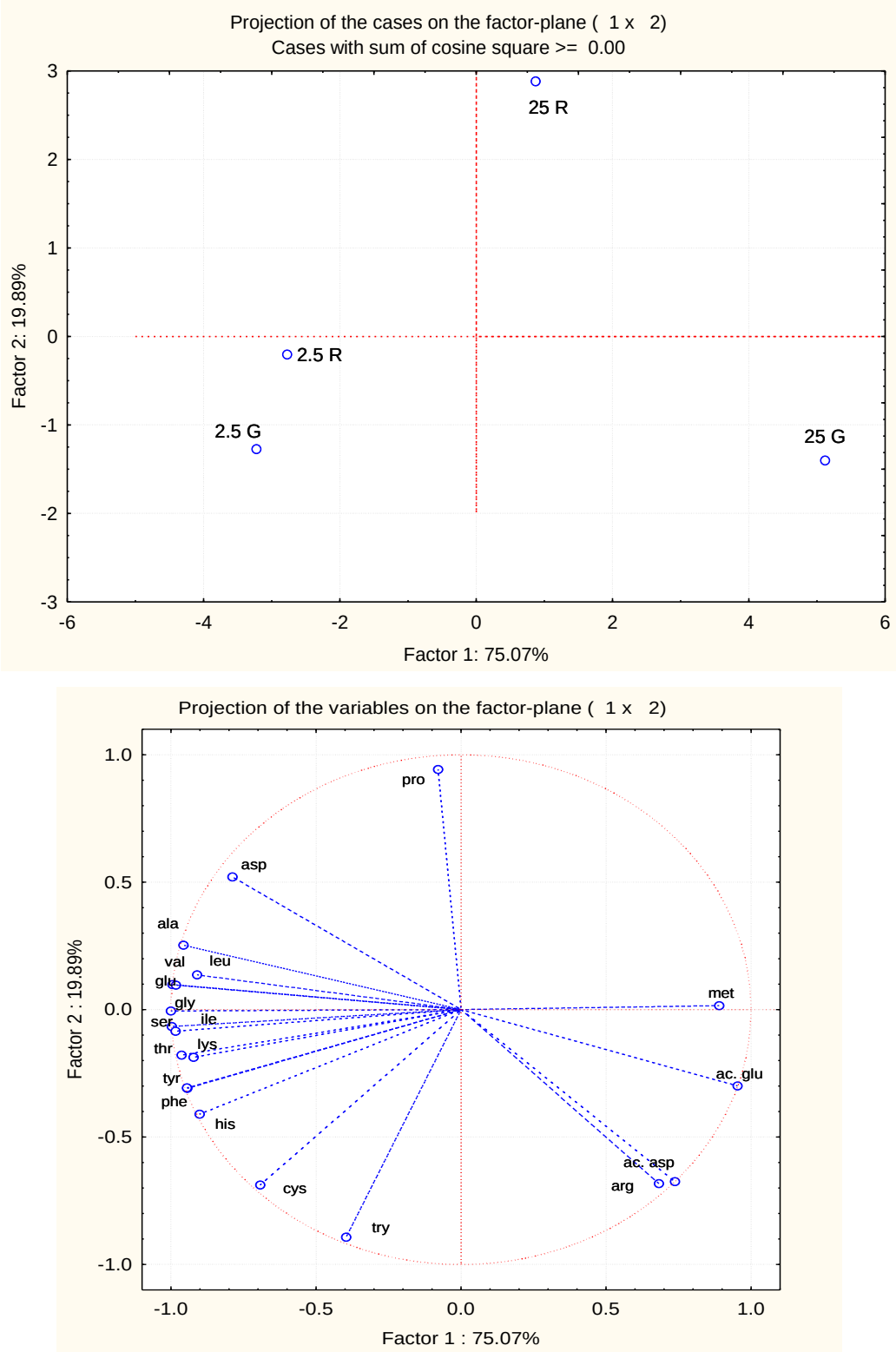

Figure 6.14: Results of Principal Component Analysis: projection of case coordinates on the sample score plot of the Factors 1 and 2 (above); PCA loading plot of the amino acids selected on the first two factors obtained from PCA (below).

The Factor 1 and the Factor 2 explain the 75.07% and 19.89% of the variability, respectively. Factor 1 clearly separated the samples with 2.5 mM of sugar from the samples

with 25 mM of sugars. Serine, histidine, glutamine, threonine, cysteine, lysine and tryptamine showed a lower consumption in the samples with 2.5 mM of sugars and were all negatively correlated with the Factor 1, differently from methionine and glutamic acid, highly positively correlated with Factor 1. The Factor 2 was negatively correlated with aspartate and arginine, the latter being consumed in lower amount by the sample containing 25 mM glucose. By contrast, the same Factor was positively correlated with proline, which showed higher accumulation in the samples containing ribose, especially 25 mM.

The two factors were able to well separate the samples in relation to the sugar content. Also the samples containing 25 mM of sugar resulted well discriminated. A lower discrimination was obtained for the media containing 2.5 mM of sugars, mainly attributable to the higher proline accumulation in the sample ribose 25 mM.

	Ethanol	**Acetoin**	**2,3-butanediol**	**Glucose**	**Ribose**
48h_2.5 G	0.01	0.10	0.01	-	-
48h_2.5 R	0.01	0.15	0.01	-	-
48h_25 G	0.03	0.14	0.06	0.14	-
48h_25 R	0.01	0.21	0.19	-	0.15

Table 6.4.: Concentration (expressed as mM) of some metabolic compounds detected by ^{1}H-NMR in *L. sakei* Chr82 cells after 48 hours of incubation at 30°C in different conditions.

In addition to the amino acid content, the ^{1}H-NMR protocol applied allowed the detection of other metabolic compounds. In particular, the concentration of sugars, which resulted completely depleted in the media in which they were added at 2.5mM, while in the samples added with 25 mM, small residual quantities (approx. 0.15 mM) were detected. Also, ethanol was detected in extremely low amounts, indicating that the secondary metabolic pathways activated by *L. sakei* Chr82 were mainly addressed towards the production of acetic acid which allow the production of energy rather than the regeneration of reduced NADH. The production of acetoin and 2,3-butanediol was higher in the samples containing higher sugar concentration, particularly in the sample added with ribose.

6.4. CONCLUSIONS

The results obtained in this part of the research increased the knowledge on the physiological and metabolic responses of *L. sakei* in relation to different sugar amounts. Indeed, the combined use of HPLC and NMR approaches allowed to better elucidate the consumption of amino acids and the resulting metabolites produced during incubation.

As expected, higher concentrations of glucose or ribose induced higher growth performances, acidification of growth medium and accumulation of lactic and acetic acids.

Flow cytometric analysis evidenced different physiological adaptation to the conditions: in fact, even if cells grown on glucose at high concentration had a high viability, the same sugar in low amounts induced the presence of dead cells, while ribose determined higher percentage of injured cells but only few cells were recognized as dead, also when this pentose was present at 2.5 mM.

The analysis of amino acids confirmed the rapid depletion of specific amino acids, mainly arginine, whose consumption was higher in the presence of ribose and resulted in the production of ornithine. Other amino acids highly consumed by this strain were serine, asparagine, glutamine, histidine. Since the HPLC protocol adopted was not able to separate these metabolites (co-elution of serine+asparagine and glutamine+histidine), NMR analysis in this case was helpful to discriminate the single metabolites, allowing to evidence a higher consumption of serine, especially when sugars were present at optimal concentration. This consumption of serine confirmed other findings reported in literature for LAB, i.e. the use of this amino acid as a source of pyruvate, which can then be used to produce energy through secondary pathways (Ganzle, 2015).

The high-resolution nuclear magnetic resonance (^{1}H-NMR) analysis performed on the same samples with the aim to set up a fast method to simultaneously quantify amino acids, sugars and organic acids was successful. This approach resulted indeed suitable and very promising to evaluate the metabolic response of *L. sakei* in terms of consumption and accumulation of specific metabolites.

In conclusion, this study evidenced a different metabolic and physiological behaviour of *L. sakei* in relation to the different carbon source as active response to harsh conditions.

This information can be helpful to optimize the use of this species as starter culture for the industrial production of fermented sausages, since stressful condition can affect the

microbial technological performances or induce the activation of specific metabolic pathways, whose final products can have a significant impact on the sensorial features of the fermented sausages obtained.

CHAPTER 7

CONCLUSIONS

In the last decades the industry of fermented meats had to face drastic challenges related to several factors dependent on nutritional aspects, technological innovations, social habits.

From the nutritional point of view, the main driver of innovation regarded the diminution of salt content to the meat batter before casing. This diminution (today salt additions of 2.2-2.5% are common compared to 3.0-3.5% used until 25-20 years ago) determined dramatic consequences on some textural properties of fermented sausages as well as on the microbial population activities in fermented meats. In addition to the reduction of NaCl, more recently the request of consumers to eliminate nitrite and nitrate from sausages, due to health reasons, has been responsible for a further complication of the microbiological situation.

The need to face these challenges determined a wide diffusion of the use of starter cultures, coupled with the addition of sugars, as a necessary tool for guaranteeing the safety and the organoleptic quality of these products.

Nevertheless, in Italy the use of starter cultures has been not always accompanied by an adequate preparation of the personnel responsible for the processes, both in small and big industries. In fact, the adjunct of starter cultures is always seen as an additive rather than a microbial population which has to interact with the environment and with the other wild microorganisms with the aim of driving a correct fermentation and ripening of the final product.

The main criteria used for the selection of starter cultures, usually added as mixtures of lactobacilli (*L. sakei*, *L. curvatus*, *L. plantarum*, *P. pentosaceus*, *P. acidilactici*) and staphylococci (*S. xylosus* and *S. carnosus*) are the rapid fermentation of the sugars present (with the consequent pH decrease) and the ability to maintain the viability and the control of sausage during ripening. The success in this colonization depends in first instance on the ability of the culture to overcome the microorganisms present in the meat mixture and this is related to the competitiveness of these cells and its number, given that, differently from many other fermented foods (such as dairy products), minced meat cannot be treated for the reduction of the initial microorganisms.

The starting point of this PhD thesis was a case study in which a not proper use of starter cultures brought to obtain sausages with several defects both organoleptic and hygienic. The main hurdle encountered by the starter cultures was the high number of cells present in the raw materials. The reasons of this microbial concentrations rely on the use of meat defrosted

characterized by counts rather high, about 6 log cfu/g. This has been probably caused by a incorrect handling of the meat before thawing or during defrosting. Starter cultures added to the concentration suggested by the supplier (approx. 6 log cfu/g) were not able to compete with these cells already adapted to the environmental conditions. The ideal ratio between selected cultures and wild microorganism is 100:1. Competitive starters can prevail at 10:1, even if the process parameters have to be strictly controlled. This is the reason why a responsible use of selected cultures requires the personnel trained and aware that they must not be considered an "additive" but living cells interacting with the environment.

A failure of the activity of starter cultures such as the case described here resulted in the presence of aroma defects, mainly attributed to the high concentration of isovaleric acid, and in the production of alarming contents of biogenic amines.

The case study described suggests a deeper study of the characteristics of the starter cultures in relation to their colonizing potential and, also, to their capability to contribute to the biological processes important for the ripening and the formation of the organoleptic properties of sausages.

In particular, our study was focused of the characteristics of *L. sakei*, the main LAB species used as starter culture in fermented sausages. In this PhD thesis we do not consider the role of staphylococci, which are important especially in relation to the aroma profile formation. However, *L. sakei* plays a crucial role due to its ability in colonizing meat environments and to its capacity to maintain the dominance throughout all the ripening (and commercialization) period, avoiding the possibility of growth of undesired microorganisms, both pathogens and spoilers. *L. sakei* is a peculiar LAB species highly adapted to meat environment due to its specific requirements in amino acid needs and the capacity of metabolize pentoses such as ribose, which can be found in meat, where the presence of fermentable hexoses is scarce. In addition, it shows a long survival (up to several weeks) in this environment whereas, if grown of culture media (such as MRS), it rapidly dies after reaching the stationary phase.

In this framework, we focused our attention of the effects of the available fermentable sugars (ribose and glucose) on the growth of members of this species and the collateral metabolism of amino acids, with particular attention to their use as energy source.

The studies carried out demonstrated in first instance that the presence of the specific sugars determined the activation not only of the metabolic pathways necessary for homo- and heterofermentative fermentation, but was able to activate also other pathways that, in first instance, may be useful for the survival of the species in nutritional harsh conditions and, as secondary effect, may be important for the determination of the aromatic profile of a fermented sausage. In addition, in the second part of this PhD thesis, we confirmed the extreme variability of this species. This variability was already evidenced by genomic studies and by screening carried out by several researches, but, in this case, we clearly evidenced the presence of different phenotypes of the species in relation to environmental conditions. In particular, the intra species variability was studied by evaluating the activity of resting cells suspended in a defined medium in which only amino acids were added as nitrogen source.

Independently of this variability, the metabolic activities of the strains were affected both by the type and amount of sugar added. The presence of ribose enhanced pyruvate metabolism with consequences on the energy balance but also on the production of compound such as diacetyl and acetoin, which can exert a role in the flavor profile. Among amino acids, determined through an HPLC protocol, arginine was highly depleted by almost all the strains (with the exception of the type strain) especially in the absence of sugars added and when the strains were precultivated on ribose, confirming that the ADI pathway is a crucial metabolic mechanism able to allow the survival of the species in the absence of fermentable sugars. However, some other amino acids were depleted in high amounts under specific conditions. This is the case of methionine and serine+asparagine (not separated by the HPLC protocol) which can be an additional source of pyruvate used for producing energy *via* POX or PDH. The adoption of this strategy is demonstrated by the excess of production of acetic acid and, in some cases, formic acid.

These behaviors represented the metabolism of resting cells in the defined medium.

In the last part of the PhD thesis the attention was focused on two objectives. The first was the evaluation of performances of growing cells of one of the strains in a medium containing different amounts of ribose or glucose and the second was to set up a NMR protocol aimed to give a reliable overview of the metabolomic of the strain.

The NMR approach was successful and allowed to highlight a presence of amino acids highly correlated with the HPLC data. In addition, the method permitted the separation of the amino acids not resolved by the HPLC protocol (alanine+asparagine, histidine+glutamine). Finally, the same analysis allowed to detect also relevant information about molecules such as organic acids, ethanol, C4 compounds which gave a reliable picture of the metabolic strategy adopted by the strain in relation to the growing conditions. Some differences were observed between growing and resting cells, but the presence of an efficient metabolism of pyruvate obtained from other sources (alanine in first instance) was confirmed. In addition, the passage from the homo- (with glucose) and heterofermentative (with ribose) to mixed acid fermentation was demonstrated.

In conclusion, this PhD thesis demonstrated the importance of a deeper study of LAB starter cultures used for meat fermentations, mainly in relation to the biodiversity characterizing the species *L. sakei*. The genomic diversity of the species had been already demonstrated by other Authors, and this work was addressed to understand the phenotypic consequences of this diversity. The final and applicative results of this study are crucial for the optimization of the use of starter cultures through pre-culture conditions and cell viability, improving their competitiveness. In addition, the exploitation of the differences will allow to identify specific strains for specific conditions, permitting a use of the starter cultures for safer products and the characterization and differentiation of the final sausages through a more conscious use of these bacteria.

BIBLIOGRAPHY

- ✓ Alan, Y., Topalcengiz, Z., Dığrak, M. (2018). Biogenic amine and fermentation metabolite production assessments of Lactobacillus plantarum isolates for naturally fermented pickles. LWT - Food Science and Technology, 98, 322-328;
- ✓ Allison G.E., Ahn C., Stiles M. E., Klaenhammer T.R. (1995). Utilization of the leucocin A export system in *Leuconostoc gelidum* for production of a *Lactobacillus* bacteriocin. *FEMS Microbiology Letters*, 131: 87-93;
- ✓ Ammor S., Dufour E., Zagorec M., Chaillou S., and Chevallier I. (2005). Characterization and selection of *Lactobacillus sakei* strains isolated from traditional dry sausage for their potential use as starter cultures. Food Microbiol. 22, 529-538;
- ✓ Andersen L. (1998). Fermented, dry sausages with the admixture of probiotic cultures. In *Proceedings, 44th international congress on meat science and technology*, Barcelona. (pp.826-827). IRTA/EUROCARNE;
- ✓ Anifantaki K., Metaxopoulos J., Kammenou M., Drosinos E.H., Vlassi M. (2002). The effect of smoking, packaging and storage temperature on the bacterial greening of frankfurters caused by *Leuconostoc mesenteroides* subsp. *Mesenteroides*. *Italian Journal of Food Science* 14 (2): 135-144;
- ✓ Ansorena D., Gimeno O., Astiasarán I. and Bello J. (2001). Analysis of volatile compounds by GC–MS of a dry fermented sausage: Chorizo de Pamplona. *Food Research International* 34: 67-75;
- ✓ Ansorena D., Astiasaran I. (2007). Functional meat products. In F. Toldrà (Ed.), *Handbook of fermented meat and poultry* (pp. 257-266). Ames, Iowa, USA: Blackwell Publishing;
- ✓ Aquilanti L., Garofalo C., Osimani A., Clementi F. (2016). Ecology of lactic acid bacteria and coagulase negative cocci in fermented dry sausages manufactured in Italy and other Mediterranean countries: an overview. *International Food Research Journal* 23 (2): 429-445;
- ✓ Axelsson L. (2004). Lactic acid bacteria: Classification and physiology. In *Lactic Acid Bacteria: Microbiology and Functional Aspects*, 3rd ed., edited by S. Salminen, A. Ouwehand and A. Von Wright. New York: Marcel Dekker;

- ✓ Ayensa Aguirre M. G. (1993). Estudio de la evolucion del proceso de anchoado de sardina (*Sardina pilchardus, Walb*). *Alimenta* 248, 71-85;
- ✓ Barbieri, F., Montanari, C., Gardini, F., Tabanelli, G. (2019). Biogenic amine production by lactic acid bacteria: a review. Foods, 8, 17;
- ✓ Barbosa M.S., Jurkiewicz C., Landgraf M., Todorov S.D., Franco B.D.G.M. (2018). Effect of proteins, glucose and NaCl on growth, biosynthesis and functionality of bacteriocins of *Lactobacillus sakei* subsp. *sakei* 2a in foods during storage at 4 °C: Tests in food models. LWT- *Food Science and Technology*, 95:167-171;
- ✓ Bardocz S., Grant G., Brow D. S., Ralph A., Pusztai A. (1993). Polyamines in food-implications for growth and health. *Journal of Nutritional Biochemistry* 4: 66-71;
- ✓ Bargossi E., Tabanelli G., Montanari C., Lanciotti R., Gatto V., Gardini F. (2015). Tyrosine decarboxylase activity of enterococci grown in media with different nutritional potential: tyramine and 2-phenylethylamine accumulation and tyrDC gene expression. *Frontiers Microbiology* 6, 259;
- ✓ Barrière C., Bruckner R., Centeno D. and Talon R. (2002). Characterization of the katA gene encoding a catalase and evidence for at least a second catalase activity in *Staphylococcus xylosus*, bacteria used in food fermentation. *FEMS Microbiology Letters* 216 (2): 277-283;
- ✓ Beck H., Hansen A., Lauritsen F. (2002). Metabolite production and kinetics of branched-chain aldehyde oxidation in *Staphylococcus xylosus*. *Enzyme and Microbial Technology*, 31: 94-101;
- ✓ Belfiore C., Fadda S., Raya R., Vignolo G. (2013). Molecular basis of the adaption of the anchovy isolate *Lactobacillus sakei* CRL1756 to salted environments through a proteomic approach. *Food Research International* 54, 1334-1341;
- ✓ Biagioli, M., Laghi, L., Carino, A., Cipriani, S., Distrutti, E., Marchianò, S., Parolin, C., Scarpelli, P., Vitali, B., Fiorucci, S. (2017). Metabolic Variability of a Multispecies Probiotic Preparation Impacts on the Anti-inflammatory Activity. Frontiers in Pharmacology, 8, 505.
- ✓ Bohme H.M., Mellett F.D., Dicks L.M.T., Basson D.S. (1996). Production of salami from ostrich meat with strains of *Lactobacillus sakei*, *Lactobacillus curvatus* and *Micrococcus*. *Meat Science*, 44: 173-180;

- ✓ Borys A. (1978). Chemical composition of carbonyl fraction of smoke obtained from some kind of wood. *Roczniki Instytutu Przemysłu Miesnego I Tłuszczowego* 25: 157-182 (in Polish);
- ✓ Boulton A.A., Cookson B., Paulton R. (1970). Hypertensive crisis in a patient on MAOI antidepressants following a meal of beef liver. *Canadian Medical Association Journal* 102: 1394-1395;
- ✓ Bover-Cid S., Hugas M., Izquierdo-Pulido M. and Vidal-Carou M. (2000a). Reduction of biogenic amine formation using a negative amino acid-decarboxylase starter culture for fermentation of "Fuet" sausages. *Journal of Food Protection* 63: 237-243;
- ✓ Bover-Cid S., Izquierdo-Pulido M. and Vidal-Carou M. (2001). Effectiveness of a *Lactobacillus sakei* starter culture in the reduction of biogenic amine accumulation as a function of the raw material quality. *Journal of Food Protection* 64: 367-373;
- ✓ Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J., Holmes, S. P. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. Nature Methods, 13, 581-583;
- ✓ Carballo, J. (2012). The role of fermentation reactions in the generation of flavor and aroma of foods. In *Fermentation: Effects on Food Properties*, eds. B.M. Mehta, A. Kamal-Eldin, R.Z. Iwanski, 51-87. Boca Raton: CRC Press;
- ✓ Casaburi A., Aristoy M., Cavella S., Di Monaco R., Ercolini D., Toldra F. and Villani F. (2007). Biochemical and sensory characteristics of traditional fermented sausages of vallo di diano (Southern Italy) as affected by use of starter cultures. *Meat Science* 76: 295-307;
- ✓ Cavadini C., Hertel C., Hammes W.P. (1998). Application of lysostaphin-producing lactobacilli to control staphylococcal food poisoning in meat products. *Journal of Food Protection*, 61: 419-424;
- ✓ Chaillou S., Champomier-Verges M., Cornet M., Crutz-Le Coq A., Dudez A., Martin V., Beaufi S., Darbon-Rongere Bossy E., Loux R., Zagorec M. (2005). The complete genome sequence of the meat-borne lactic acid bacterium *Lactobacillus sakei* 23K. *Nature Biotechnology* 23: 1527-1533;

- ✓ Chaillou S., Daty M., Baraige F., Dudez A.M., Anglade P., Jones R., Alpert C.A., Champomier-Vergès M.C., Zagorec M. (2009). Intra-species genomic diversity and natural population structure of the meat borne lactic acid bacterium *Lactobacillus sakei*. *Applied Environment Microbiology*, 75:970-980;
- ✓ Chaillou, S., Lucquin, I., Najjari, A., Zagorec, M., and Champomier-Vergès, M.C. (2013). Population genetics of *Lactobacillus sakei* reveals three lineages with distinct evolutionary histories. PLoS One 8, e73253.
- ✓ Champomier-Vergès M.C., Zuñiga M., Morel-Deville F., Perez-Martinez G., Zagorec M. (1999). Relationship between arginine degradation, pH and survival in *Lactobacillus sakei. FEMS Microbiology Letters* 180: 297-304;
- ✓ Champomier-Vergès, M.C., Chailou, S., Cornet, M., and Zagorec, M. (2001). *Lactobacillus sakei*: recent developments and future prospects. Res. Microbiol. 152, 839-848.
- ✓ Chiaramonte F., Blugeon S., Chaillou S., Langella P., Zagorec M. (2009). Behavior of the meat-borne bacterium *Lactobacillus sakei* during its transit through the gastrointestinal tracts of axenic and conventional mice. *Applied and Environmental Microbiology*, 75: 4498-4505;
- ✓ Chizzolini R., Novelli E. and Zanardi E. (1998). Oxidation in traditional Mediterranean meat products. *Meat Science* 49 (1): S87-S99;
- ✓ Claesson M.J., van Sinderen D., O'Toole P.W. (2007). The genus *Lactobacillus* - a genomic basis for understanding its diversity. FEMS *Microbiology Letters*, 269: 22-28;
- ✓ Cocconcelli, P.S., and Fontana, C. (2010). Starter cultures for meat fermentation, in Handbook of meat processing, ed. F. Toldrá (Ames: Blackwell Publishing), 199-218.
- ✓ Cocolin L., Urso R., Rantsiou K., Cantoni C., Comi G. (2006). Dynamics and characterization of yeasts during natural fermentation of Italian sausages. FEMS *Yeast Research* 6 (5): 692-701;
- ✓ Coffey A., Ryan M., Ross R.P., Hill C., Arendt E., Schwarz G. (1998). Use of a broad-host-range bacteriocin-producing *Lactococcus lactis* transconjugant as an alternative starter for salami manufacture. *International Journal of Food Microbiology*, 43: 231-235;

- ✓ Coll, J., Leal, J.A. (1972). Utilization of L-leucine as nitrogen source by fungi. Transactions of the British Mycological Society, 59, 107-114.
- ✓ Dainty R.H., Blom H. (1995). Flavour chemistry of fermented sausages. In G. Campbell-Platt, & P. E. Cook, *Fermented meats* (pp.176-193). London: Blackie Academic and Professional;
- ✓ Davis, N. M., Proctor, D.M., Holmes, S.P., Relman, D.A., Callahan, B. J. (2018). Simple statistical identification and removal of contaminant sequences in marker-gene and metagenomics data. Microbiome, 6, 226.
- ✓ De Vuyst L., and Vandamme E. J., (1994). Lactic acid bacteria and bacteriocins: Their practical importance. In L. De Vuyst, E.J. Vandamme (eds) *Bacteriocins of lactic acid bacteria*, pp 1-11. Blackie, Glasgow;
- ✓ De Vuyst L. (2000). Technology aspects related to the application of functional starter cultures. Food Technology and Biotechnology 38 (2): 105-112;
- ✓ Demeyer D.I., Verplaetse A., Gistelink M. (1986). Fermentation of meat: an integrated process. *Belgian Journal of Food Chemistry and Biotechnology* 41: 131-140;
- ✓ Drosinos H., Paramithiotis S., Kolovos G., Tsikouras J. and Metaxopoulos J. (2007). Phenotypic and technological diversity of lactic acid bacteria and staphylococci isolated from traditionally fermented sausages in Southern Greece. *Food Microbiology* 24: 260-270;
- ✓ Duhutrel, P., Bordat, C., Wu, T.D., Zagorec, M., Guerquin-Kern, J.L., and Champomier-Vergès, M.C. (2010). Iron sources used by the non pathogenic lactic acid bacterium Lactobacillus sakei revealed by electron energy loss spectroscopy and secondary ions mass spectrometry. Appl. Environ. Microbiol. 76, 560-565.
- ✓ Erkkila S. and Petaja E. (2000). Screening of commercial meat starter cultures at low pH and in the presence of bile salts for potential probiotic use. *Meat Science* 55: 297-300;
- ✓ Erkkilä S., Suihko M., Eerola S., Petaja E. and Mattila-Sandholm T. (2001). Dry sausage fermented by *Lactobacillus rhamnosus* strains. *International Journal of Food Microbiology* 64: 205-210;

- ✓ Estrada-Muñoz R., Boyle E.A.E., Marsden J.L. (1998). Liquid smoke effects on *Escherichia coli* O157:H7, and its antioxidant properties in beef products. *Journal of Food Science* 63 (1): 150-153;
- ✓ Fadda S., Sanz Y., Vignolo G., Aristoy M.C., Oliver G., Toldra F. (1999). Hydrolysis of pork muscle sarcoplasmic proteins by *Lactobacillus sakei* and *Lactobacillus curvatus*. *Applied and Environmental Microbiology*. 65: 578-584;
- ✓ Fadda S., Oliver G. and Vignolo G. (2002). Protein degradation by *Lactobacillus plantarum* and *Lactobacillus casei* in a sausage model system. *Journal of Food Science* 67: 1179-1183;
- ✓ Fadda S., Anglade P., Baraige F., Zagorec M., Talon R., Vignolo G., Champomier-Vergès M.C. (2010). Adaptive response of *Lactobacillus sakei* 23K during growth in the presence of meat extracts: A proteomic approach. *International Journal of Food Microbiology* 142, 36-43.
- ✓ Farber J.M., Daley E., Holley R., Usborne W.R. (1993). Survival of *Listeria monocytogenes* during the production of uncooked German, American and Italian style fermented sausages. *Food Microbiology*, 10: 123-132;
- ✓ Fernández M., Kleerebezem M., Kuipers O.P., Siezen R.J., Van Kranenburg R. (2002). Regulation of the metC-cysK operon, involved in sulfur metabolism in *Lactococcus lactis*. *Journal of Bacteriology*, 184: 82-90;
- ✓ Fernández M., Zúñiga M. (2006). Amino acid catabolic pathways of lactic acid bacteria. *Critical Reviews in Microbiology* 32: 155-183;
- ✓ Fernández, M., Kleerebezem, M., Kuipers, O.P., Siezen, R.J., and van Kranenburg, R. (2002). Regulation of the metC-cysK operon, involved in sulfur metabolism in Lactococcus lactis. J. Bacteriol. 184, 82-90.
- ✓ Fiehn, O. (2002). Metabolomics – the link between genotypes and phenotypes. Plant Molecular Biology, 48, 155-171.
- ✓ Flores M. (2018). Understanding the implications of current health trends on the aroma of wet and dry cured meat products. *Meat Science*, 144: 53-61;
- ✓ Flores M., Corral S., Cano-García L., Salvador A., Belloch C. (2015). Yeast strains as potential aroma enhancers in dry fermented sausages. *International Journal of Food Microbiology* 212: 16-24;

- ✓ Flores, M., Olivares, A. (2015). Flavor. In F. Toldrá (Ed.). Handbook of fermented meat and poultry (pp. 217–225). (2nd edition). John Wiley Sons, Ltd
- ✓ Freiding, S., Ehrmann, M.A., and Vogel, R.F. (2012). Comparison of different IlvE aminotransferases in Lactobacillus sakei and investigation of their contribution to aroma formation from branched chain amino acids. Food Microbiol. 29, 205-214.
- ✓ Gaier W., Vogel R.F., Hammes W.P. (1992). Cloning and expression of the lysostaphin gene in *Bacillus subtilis* and *Lactobacillus casei*. *Letters in Applied Microbiology*, 14: 72-76;
- ✓ Galgano F., Favati F., Schirone M., Martuscelli M. and Crudele M. (2003). Influence of indigenous starter cultures on the free fatty acids content during ripening in artisan sausages produced in the Basilicata region. *Food Technology and Biotechnology* 41: 253-258;
- ✓ Gänzle, M.G. (2015). Lactic metabolism revisited: metabolism of lactic acid bacteria in food fermentations and food spoilage. Current Opinion in Food Science, 2, 106-117.
- ✓ Garcia M.L., Dominguez R., Galvez M.D., Casas C. and Selgas M.D. (2002). Utilization of cereal and fruit fibres in low fat dry fermented sausages. *Meat Science* 60: 227-236;
- ✓ Gardini F., Suzzi G., Lombardi A., Galgano F., Crudele M.A., Andrighetto C., Schirone M., Tofalo R. (2001). A survey of yeasts in traditional sausages of southern Italy. FEMS *Yeast Research* 1 (2): 161-167;
- ✓ Gardini, F., Özogul, Y., Suzzi, G., Tabanelli, G., Özogul, F. (2016). Technological factors affecting biogenic amine content in foods: A review. Frontiers in Microbiology, 7, 1218.
- ✓ Garrity G., Bell J. and Lilburn T. (2004). Taxonomic outline of the prokaryotes. In *Bergey's Manual of Systematic Bacteriology*, 2nd ed. New York: Springer-Verlag;
- ✓ Gazzetta Ufficiale, Serie Generale, N. 89 del 15/4/1995;
- ✓ Geisen R. (1993). Fungal starter cultures for fermented foods: molecular aspects. *Trends in Food Science and Technology*, 4: 251-256;
- ✓ Geyzen A. (2011). Popular discourse on nutrition, health and indulgence in Flanders, 1945-1960. *Appetite*, 56: 278-283;

- ✓ Geyzen A., Scholliers P., Leroy F. (2012). Innovative traditions in swiftly transforming foodscapes: an exploratory essay. Trends in *Food Science & Technology*, 25: 47-52;
- ✓ Gianelli, M.P., Olivares A., Flores, M. (2011). Key aroma components of a dry-cured sausage with high fat content (Sobrassada). Food Science and Technology International, 17, 63-71.
- ✓ Gimeno O., Astiasaran I. and Bello J. (2001). Calcium ascorbate as a potential partial substitute for NaCl in dry fermented sausages: Effect on colour, texture and hygienic quality at different concentrations. *Meat Science* 57: 23-29;
- ✓ Gorissen L., Raes K., De Smet S., De Vuyst L., Leroy F. (2012). Microbial production of conjugated linoleic and linolenic acids in fermented foods: technological bottlenecks. *European Journal of Lipid Science and Technology*, 114: 486-491;
- ✓ Gou P., Comaposada J., Arnau J. (2004). Moisture diffusivity in the lean tissue of dry-cured ham at different process times. *Meat Science* 67: 203;
- ✓ Gou P., Guerrero L., Gelabert J. and Arnau J. (1996). Potassium chloride, potassium lactate and glycine as sodium chloride substitutes in fermented sausages and in dry - cured pork loin. *Meat Science* 42: 37-48;
- ✓ Gozzi, G., Vannini, L., Bouchefra, A., Idoui, T., Tabanelli, G., Barbieri, F., Gardini, F., and Montanari, C. (2017). Can "El Kadid", a dried camel meat product of Algeria, be a source for new starter cultures? 3rd International Symposium on Fermented Meat, Clermont-Ferrand (France), 27-29 September 2017, Book of abstract, P-02, 30.
- ✓ Gratzer W. (2008). Terrors of the table. The curious history of nutrition. Oxford: *Oxford University Press*;
- ✓ Grunert K. G., Verbeke W., Keuglera J.O., Saeed F., Scholderer J. (2011). Use of consumer insight in the new product development process in the meat sector. *Meat Science*, 89: 251-258;
- ✓ Grunert K.G., Valli C. (2001). Designer-made meat and dairy products: consumer-led product development. *Livestock Production Science*, 72: 83-98;
- ✓ Guerrero L., Guardia M.D., Xicola J., Verbeke W., Vanhonacker F., Zakowska-Biemans S. (2009). Consumer-driven definition of traditional food products and

innovation in traditional foods. A qualitative cross-cultural study. *Appetite*, 52: 345-354;

- ✓ Guilbaud, M., Zagorec, M., Chaillou, S., and Champomier-Vergès, M.C. (2012). Intraspecies diversity of Lactobacillus sakei response to oxidative stress and variability of strain performance in mixed strains challenges. Food Microbiol. 29, 197-204.
- ✓ Haakensen M., Dobson C.M., Hill J.E., Ziola B. (2009). Reclassification of *Pediococcus dextrinicus* (Coster and White 1964) back 1978 (approved lists 1980) as *Lactobacillus dextrinicus* comb. nov., and emended description of the genus *Lactobacillus*. *International Journal of Systematic and Evolutionary Microbiology* 59 (3): 615-621;
- ✓ Halász A. (2009). Lactic acid bacteria. In: *Food Quality and Standards 3*. pp. 70-82;
- ✓ Halàsz A., Baràth A., Simon-Sarkadi L., Holzapfel W. (1994). Biogenic amines and their production by microorganisms in food. *Trends in Food Science & Technology*, 5: 42-49;
- ✓ Hammes, W.P., and Hertel, C. (1998). New developments in meat starter cultures. Meat Sci. 49, 125-138.
- ✓ Hansen E.B. (2002). Commercial bacterial starter cultures for fermented foods of the future. *International Journal of Food Microbiology* 78: 119-131;
- ✓ Hernandez-Jover T., Izquierdo-Pulido M., Veciana-Nogues M.T., Vidal-Carou, M.C. (1996). Biogenic amine sources in cooked cured shoulder pork. *Journal of Agriculture and Food Chemistry* 44, 3097–3101;
- ✓ Hertel C., Schmidt G., Fischer M., Oellers K., Hammes W.P. (1998). Oxygen-dependent regulation of the expression of the catalase gene katA of *Lactobacillus sakei* LTH677. *Applied and Environmental Microbiology*. 64: 1359-1365;
- ✓ Hiyama T., Fukui S., Kitahara K. (1968). Purification of the lactate racemase from *Lactobacillus sake*. *The Journal of biochemistry*. (Tokyo) 64: 99-107;
- ✓ Holzapfel W.H. (1997). Use of starter cultures in fermentation on a household scale. *Food Control* 8 (5-6): 241-258;

- ✓ Holzapfel W.H., Haberer P., Snel J., Schillinger U., Huisin't Veld J.H.J. (1998). Overview of gut flora and probiotics. *International Journal of Food Microbiology*, 41: 85-101;
- ✓ Holzapfel W.H. (2002). Appropriate starter culture technologies for small-scale fermentation in developing countries. *International Journal of Food Microbiology* 75 (3): 197-212;
- ✓ Hornero-Mendez D., Garrido-Fernandez A. (1997). Rapid high performance liquid chromatography analysis of biogenic amines in fermented vegetable brines. *Journal of Food Protection* 60: 414-41;
- ✓ Hugas, M., Garriga, M., Aymerich, T., and Monfort, J.M. (1993). Biochemical characterization of lactobacilli from dry fermented sausages. Int. J. Food Microbiol. 18, 107-113.
- ✓ Hughes M., Kerry P., Arendt E., Kenneally P., McSweeney P. and O'Neill E. (2002). Characterization of proteolysis during the ripening of semi-dry fermented sausages. *Meat Science* 62: 205-216;
- ✓ Ibanez C., Quintanilla L., Irigoyen A., Garcia-Jalon I., Cid C., Astiasaran I. and Bello J. (1995). Partial replacement of sodium chloride with potassium chloride in dry fermented sausages: Influence on carbohydrate fermentation and the nitrosation process. *Meat Science* 40: 45-53;
- ✓ Ibe A., Nishina T., Kasai N. (1992). Bacteriological properties of and amine-production conditions for tyramine and histamine-producing bacterial strains isolated from soybean paste (miso) starting materials. Japan. *Journal of Toxicology and Environmental Health* 38: 403-409;
- ✓ Igarashi K., Ito K., Kashiwagi K. (2001). Polyamine uptake systems in *Escherichia coli*. *Research in Microbiology* 152: 271-278;
- ✓ Iino, T., Uchimura, T., and Komagata, K. (2003). The effect of sodium acetate on the activity of L- and D-lactate dehydrogenases in Lactobacillus sakei NRIC 1071T and other lactic acid bacteria. J. Gen. Appl. Microbiol. 49, 51-58.
- ✓ Imre L. (1974). Drying of salami sorts. In *Handbook of Drying* (in Hungarian) edited by L. Imre. Budapest: Müszaki Kiadó (Publisher of the Technics);
- ✓ Incze K. (1998). Dry fermented sausages. *Meat Science* 49 (1): S169-S177;

- ✓ Incze K. (2004). Dry and semi - dry sausages. In *Encyclopedia of Meat Sciences*, edited by W.K. Jensen, C. Devine, and M. Dikeman. London: Elsevier Academic Press;
- ✓ Irmler S., Bavan T., Oberli A., Roetschi A., Badertscher R., Guggenbühl B., Berthoud H. (2013). Catabolism of serine by *Pediococcus acidilactici* and *Pediococcus pentosaceus*. *Applied and Environmental Microbiology*, 79:1309-1315;
- ✓ Janssens M., Myter N., De Vuyst L., Leroy, F. (2012). Species diversity and metabolic impact of the microbiota are low in spontaneously acidified Belgian sausages with an added starter culture of *Staphylococcus carnosus*. *Food Microbiology*, 29: 167-177;
- ✓ Jay J.M. (1996). Microorganisms in fresh ground meats: the relative safety of products with low versus high numbers. *Meat Science*, 43: 59-66;
- ✓ Jørgensen L.V., Huss H.H., Dalgaard P. (2000). The effect of biogenic amine production by single bacterial cultures and metabiosis on cold-smoked salmon. *Journal of Applied Microbiology* 89: 920-934;
- ✓ Kalac P., Svecova S., Pelikanova T. (2002). Levels of biogenic amines in typical vegetable products. *Food Chemistry* 37, 349-351;
- ✓ Katagiri H., Kitahara K., Fukami K. (1934). The characteristics of the lactic acid bacteria isolated from moto, yeast mashes for sake´ manufacture. IV. Classification of the lactic acid bacteria. *Bulletin of the Agricultural Chemical Society of Japan*, 10:156-157;
- ✓ Kearney L., Upton M. and McLoughlin A. (1990). Meat fermentations with immobilized lactic acid bacteria. *Applied Microbiology and Biotechnology* 33: 648-651;
- ✓ Kenneally M., Fransen G., Grau H., O'Neill E. and Arendt K. (1999). Effects of environmental conditions on microbial proteolysis in a pork myofibril model system. *Journal of Applied Microbiology* 87: 794-803;
- ✓ Kłossowska B.M. (1979). The use of combined gas chromatography – mass spectrometry for the identification of aliphatic carboxylic acids in smoke produced from two species of wood. *Acta Alimentaria Polonica* 5(2): 301–310 (in Polish);

- ✓ Kolodziejska I., Niecikowska C., Sikorski Z.E. (1994). Dimethylamine and formaldehyde in cooked squid (I&x argentinus) muscle extract and mantle. F*ood Chemistry* 50: 281-283;
- ✓ Konings W. N., Lolkema J. S., Bolhuis H., Van Veen H. W., Poolman B., e Driessen A. J. M. (1997). The role of transport processes in survival of lactic acid bacteria. *Antonie Leeuvenhoek* 71: 117-128;
- ✓ Krizek A.R., Smith J.S., Phebus R.K. (1995). Biogenic amine formation in fresh vacuum-packaged beef stored at -2°C and 2°C for 100 days. J*ournal of Food Protection* 58: 284-288;
- ✓ Kunene N.F., Geornaras I., von Holy A., Hastings J.W. (2000). Characterization and determination of origin of lactic acid bacteria from a sorghum-based fermented weaning food by analysis of soluble proteins and amplified fragment length polymorphism fingerprinting. *Applied and Environmental Microbiology*. 66: 1084-1092;
- ✓ Kurko V.I. (1966). On the role and importance of individual smoke phenols in inhibiting the oxidative deterioration of smoked meats. *XII European Congress of Meat Research Workers*, Sandefjord;
- ✓ Laghi, L., Versari, A., Marcolini, E., Parpinello, G.P. (2014). Metabonomic Investigation by ^{1}H-NMR to Discriminate between Red Wines from Organic and Biodynamic Grapes. Food and Nutrition Sciences, 5, 52-59.
- ✓ Landete J. M., de las Rivas B., Marcobal A., Muñoz R. (2008). Updated molecular knowledge about histamine biosynthesis by bacteria. *Critical Reviews in Food Science and Nutrition* 48: 697-714;
- ✓ Langella P., Zagorec M., Ehrlich S.D., Morel-Deville F., (1996). Intergeneric and intrageneric conjugal transfer of plasmids pAMβ1, pIL205 and pIP501 in *Lactobacillus sake*. *FEMS Microbiology Letters* 139: 51-56;
- ✓ Larrouture, C., Ardaillon, V., Pepin, M., Montel, M.C. (2000). Ability of meat starter culture to catabolize leucine and evaluation ofthe degradation products by using an HPLC method. Food Microbiology 17, 563–570.
- ✓ Larrouture-Thiveyrat, C., Montel, M.C. (2003). Effects of environmental fac-tors on leucine catabolism by *Carnobacterium piscicola*. International Journal of Food

Microbiology 81, 177-184.

- ✓ Lauret R., Morel-Deville F., Berthier F., Champomier-Vergès M.C., Postma P.W., Ehrlich S.D., Zagorec M. (1996). Carbohydrate Utilization in *Lactobacillus sake*. *Applied and Environmental Microbiology* 62: 1922-1927;
- ✓ Lebert I., Leroy S., Giammarinaro P., Lebert A., Chacornac J.P., Bover-Cid S., Vidal-Carou M.C. and Talon R. (2007). Diversity of microorganisms in the environment and dry fermented sausages of small traditional French processing units. *Meat Science* 76: 112-122;
- ✓ Lee, J.H., Jin, Y.H., Park, Y.K., Yun, S.J., Mah, J.H. (2019). Formation of biogenic amines in *Pa* (Green onion) Kimchi and Gat (Mustard leaf) Kimchi. Foods, 8, 109.
- ✓ Leistner L. (1999). Combined methods for food preservation. In M. S. Rahman, *Handbook of food preservation* (pp. 427-485). New York: Dekker;
- ✓ Leroy F., Verluyten J. and De Vuyst L. (2006). Functional meat starter cultures for improved sausage fermentation. *International Journal of Food Microbiology* 106: 270-285;
- ✓ Leroy, F., Scholliers, P., Amilien, V. (2015). Elements of innovation and tradition in meat fermentation: Conflicts and synergies. International Journal of Food Microbiology, 212, 2-8.
- ✓ Lindsay H. (2012). Food representations. In P. Erdkamp (Ed.), *A cultural history of food in antiquity* (pp. 163-179). London & New York: Berg Publishers;
- ✓ Liu, S.Q., Holland, R., McJarrow, P., and Crow, V.L. (2003). Serine metabolism in Lactobacillus plantarum. Int. J. Food Microbiol. 89, 265-273.
- ✓ Lo R., Turner M.S., Barry D.G., Sreekumar R., Walsh T.P., Giffard P.M. (2009). Cystathionine g-lyase is a component of cystine-mediated oxidative defense in *Lactobacillus reuteri* BR11. *Journal of Bacteriology*, 191:1827-1837.
- ✓ Lonvaud-Funel A. (2001). Biogenic amines in wines: role of lactic acid bacteria. *FEMS Microbiology Letters* 199: 9-13;
- ✓ López-Dıáz T.M., Santos J.A., Garcıá -López M.L., Otero A. (2001). Surface mycoflora of a Spanish fermented meat sausage and toxigenicity of *Penicillium* isolates. International Journal of Food Microbiology 68 (1): 69-74;
- ✓ Lorenzo, J.M., Gómez, M., Purriños, L., Fonseca, S. (2016). Effect of commercial

starter cultures on volatile compound profile and sensory characteristics of dry-cured foal sausage. Journal of the Science of Food and Agriculture, 96, 1194-1201.

- ✓ Lücke F.K. (1986). Microbiological processes in the manufacture of dry sausage and raw ham. *Fleischwirtschaft* 66: 1505-1509;
- ✓ Lücke F.K., Bruèmmer J.M., Buckenhuèskes H., Garrido Fernandez A., Rodrigo M., Smith J.E. (1990). Starter culture development. In P. Zeuthen, J.-C. Cheftel, C. Eriksson, T. R. Gormley, P. Linko, & K. Paulus, *Processing and quality of foods. Vol. 2: food biotechnology: avenues to healthy and nutritious products* (pp. 2.11-2.36). London: Elsevier Applied Science;
- ✓ Lücke F.K. (1998). Fermented sausages. In B.J.B. Wood, *Microbiology of fermented foods*, (2nd ed., pp. 441-483). London: Blackie Academic and Professional;
- ✓ Lücke K. (2000). Utilization of microbes to process and preserve meat. *Meat Science* 56: 105-115;
- ✓ Magistà D., Susca A., Ferrara M., Logrieco A.F., Perrone G. (2017). *Penicillium* species: crossroad between quality and safety of cured meat production. *Current Opinion in Food Science*17: 36-40;
- ✓ Magrinya N., Bou R., Tres A., Rius N., Codony R., Guardiola F. (2009). Effect of tocopherol extract, *Staphylococcus carnosus* culture, and celery concentrate addition on quality parameters of organic and conventional dry-cured sausages. Journal of Agricultural and Food Chemistry, 57: 8963-8972;
- ✓ Maijala R., Eerola S., Lievonen S., Hill P., Hirvi T. (1995). Formation of biogenic amines during ripening of dry sausages as affected by starter culture and thawing of raw materials. *Journal of Food Science*, 60: 1187-1190;
- ✓ Malleret, C., Lauret, R., Dusko Ehrlich, S., Morel-Deville, F., and Zagorec, M. (1998). Disruption of the sole ldhl gene in Lactobacillus sakei prevents the production of both L- and D-lactate. Microbiology 144, 3327-3333.
- ✓ Maloney P.C. (1977). Obligatory coupling between proton entry and the synthesis of ATP by *Streptococcus lactis. Journal of Bacteriology* 132: 564-575;
- ✓ Mann N. (2000). Dietary lean red meat and human evolution. *European Journal of Nutrition* 39: 71-79;

- ✓ Marcobal A., De Las Rivas B., Landete J. M., Tabera L., Muñoz R. (2012). Tyramine and phenylethylamine biosynthesis by food bacteria. C*ritical Reviews in Food Science and Nutrition* 52: 448-467;
- ✓ Marino M., Maifreni M., Moret S., e Rondinini G. (2000). The capacity of *Enterobacteriaceae* species to produce biogenic amines in cheese. *Letters in Applied Microbiology* 31: 169-173;
- ✓ Martuscelli M., Crudele M. A., Gardini F., Suzzi G. (2000). Biogenic amine formation and oxidation by *Staphylococcus xylosus* strains from artisanal fermented sausages. *Letters in Applied Microbiology* 31: 228-232;
- ✓ Masson, F., Hinrichsen, L., Talon, R., Montel, M.C. (1999). Factors influencing leucine catabolism by a strain of *Staphylococcus carnosus*. International Journal of Food Microbiology, 49, 173-178.
- ✓ Mathieu M. (1906). Notions d'economie domestique. *Verviers*, Belgium: Hermann;
- ✓ Mauriello G., Casaburi A., Blaiotta G. and Villani F. (2004). Isolation and technological properties of coagulase negative staphylococci from fermented sausages of Southern Italy. *Meat Science* 67: 149-158;
- ✓ McLeod A., Nyquistb O.N., Snipenb L., Naterstada K., Axelsson L. (2008). Diversity of *Lactobacillus sakei* strains investigated by phenotypic and genotypic methods. *Systematic and Applied Microbiology*, 31:393-403;
- ✓ McLeod, A., Zagorec, M., Champomier-Vergès, M.C., Naterstad, K., and Axelsson, L. (2010). Primary metabolism in *Lactobacillus sakei* food isolates by proteomic analysis. BMC Microbiol. 10, 120.
- ✓ McLeod, A., Snipen, L., Naterstad, K., and Axelsson, L. (2011). Global transcriptome response in *Lactobacillus sakei* during growth on ribose. BMC Microbiol. 11, 145.
- ✓ McLeod A., Mosleth E.F., Rud I., Branco dos Santos F., Snipen L., Liland K.H., Axelsson L. (2017). Effects of glucose availability in *Lactobacillus sakei*; metabolic change and regulation of the proteome and transcriptome. *PLoS One*, 1-20.
- ✓ Mendoza E., Garcia M.L., Casas C. and Selgas M.D. (2001). Inulin as fat substitute in low fat, dry fermented sausages. *Meat Science* 57: 387-393;

- ✓ Mitchell P. (1996). Chemiosmotic coupling and energy transductions. *Glynn research*, Bodmin, Cornwall, England.
- ✓ Montanari, C., Bargossi, E., Gardini, A., Lanciotti, R., Magnani, R., Gardini, F., Tabanelli, G. (2016). Correlation between volatile profiles of Italian fermented sausages and their size and starter culture. Food Chemistry, 192, 736-744.
- ✓ Montanari, C., Gatto, V., Torriani, S., Barbieri, F., Bargossi, E., Lanciotti, R., Grazia, L., Magnani, R., Tabanelli, G., Gardini, F. (2018a). Effects of the diameter on physico-chemical, microbiological and volatile profile in dry fermented sausages produced with two different starter cultures. Food Bioscience, 22, 9-18.
- ✓ Montanari, C., Barbieri, F., Magnani, M., Grazia, L., Gardini, F., Tabanelli, G. (2018b). Phenotypic diversity of *Lactobacillus sakei* strains. Frontiers in Microbiology, 9, 2003.
- ✓ Montel M.C., Masson F., Talon R. (1998). Bacterial role in flavour development. In Proceedings, *44th international congress on meat science and technology*, Barcelona, (Vol. 1., pp. 224-233). IRTA/EUROCARNE;
- ✓ Morales R., Arnau J., Serra X., Guerrero L., Gou P. (2008). Texture changes in dry - cured ham pieces by mild thermal treatments at the end of the drying process. *Meat Science* 80: 231;
- ✓ Mosnaim A.D., Frietag F.G., Ignacia R., Salas M.A., Karoum F., Wolf M.E., Diamond S. (1996). Apparent lack of correlation between tyramine and phenylethylamine content and the occurrence of foodprecipitated migraine: re-examination of a variety of food products frequently consumed in the United States and commonly restricted in tyramine-restricted diets. *Headache Quarterly* 7, 239–249;
- ✓ Mossel D. A. A. (1971). Physiological and metabolic attitudes of microbial groups. *Journal of Applied Bacteriology* 34: 95;
- ✓ Muguerza E., Gimeno O., Ansorena D. and Astiasaran I. (2004). New formulations for healthier dry fermented sausages: a review. *Trends in Food Science and Technology* 15: 452-457;

- ✓ Nadon C.A., Ismond M.A.H., Holley R. (2001). Biogenic amines in vacuum-packaged and carbon dioxide-controlled atmosphere-packaged fresh pork stored at 1.5°C. *Journal of Food Protection* 64: 220-227;
- ✓ Nettles C. G., Barefoot S.F. (1993). Biochemical and genetic characteristics of bacteriocins of food-associated lactic acid bacteria. *Journal of Food Protection*, 56:339-356;
- ✓ Obiedzinski M., Borys A. (1977). Identification of polynuclear aromatic hydrocarbons in wood smoke. *Acta Alimentaria Polonica* 3(23)(3): 169–173 (in Polish);
- ✓ Obst M., Meding E.R., Vogel R.F., Hammes W.P. (1995). Two genes encoding the beta-galactosidase of *Lactobacillus sake*. *Microbiology* 141: 3059-3066;
- ✓ Olesen P.T., Stahnke L.H. (2000). The influence of *Debaryomyces hansenii* and *Candida utilis* on the aroma formation in garlic spiced fermented sausages and model minces. *Meat Science* 56 (4): 357-368;
- ✓ Olivares, A., Navarro, J. L., & Flores, M. (2009). Establishment of the contribution of volatile compounds to the aroma of fermented sausages at different stages of processing and storage. Food Chemistry, 115, 1464-1472.
- ✓ Olivares, A., Dryahina, K., Navarro, J.L., Smith, D., Spaněl, P., Flores, M. (2011). SPME-GC-MS versus selected ion flow tube mass spectrometry (SIFT-MS) analyses for the study of volatile compound generation and oxidation status during dry fermented sausage processing. Journal of Agricultural and Food Chemistry, 59, 1931-1938.
- ✓ Ordóňez, J. A., Hierro, E. M., Bruna, J. M., de la Hoz, L. (1999). Changes in the components of dry fermented sausages during ripening. Critical Reviews in Food Science and Nutrition, 39, 329-367.
- ✓ Ordóňez J.A., de la Hoz L. (2007). Mediterranean products. In F. Toldrà (Ed.), *Handbook of fermented meat and poultry* (pp.333-347). Ames, Iowa, USA: Blackwell Publishing;
- ✓ Papamanoli E., Tzanetakis N., Litopoulou-Tzanetaki E. and Kotzekidou P. (2003). Characterization of lactic acid bacteria isolated from a Greek dry fermented sausage in respect of their technological and probiotic properties. *Meat Science* 65: 859-867;

- ✓ Parente E., Martuscelli M., Gardini F., Grieco S., Crudele M.A. and Suzzi G. (2001). Evolution of microbial populations and biogenic amine production in dry sausages produced in Southern Italy. *Journal of Applied Microbiology* 90 (6): 882-891;
- ✓ Parolin, C., Marangoni, A., Laghi, L., Foschi, C., Ñahui Palomino, R. A., Calonghi, N., et al. (2015). Isolation of vaginal Lactobacilli and characterization of anti-Candida activity. PLoS ONE 22:10:e0131220.
- ✓ Pasini, F., Soglia, F., Petracci, M., Caboni, M.F., Marziali, S., Montanari, C., Gardini, F., Grazia, L., Tabanelli, G. (2018). Effect of fermentation with different lactic acid bacteria starter cultures on biogenic amine content and ripening patterns in dry fermented sausages. Nutrients, 10, 1497.
- ✓ Pearson A.M., Tauber F.W. (1984). *Processed meats*. *Westport*, Connecticut, USA: Avi Publishing Company;
- ✓ Pennacchia C., Ercolini D., Blaiotta G., Pepe O., Mauriello G. and Villani F. (2004). Selection of *Lactobacillus* strains from fermented sausages for their potential use as probiotics. *Meat Science* 67: 309-317;
- ✓ Pennacchia C., Vaughan E.E. and Villani F. (2006). Potential probiotic *Lactobacillus* strains from fermented sausages: Further investigations on their probiotic properties. *Meat Science* 73: 90-101;
- ✓ Pereira C., Crespo M. and Romão V. (2001). Evidence for proteolytic activity and biogenic amines production in *Lactobacillus curvatus* and *L. homohiochii*. *International Journal of Food Microbiology* 68 (3): 211-216;
- ✓ Picone, G., Laghi, L., Gardini, F., Lanciotti, R., Siroli, L., Capozzi, F. (2013) Evaluation of the effect of carvacrol on the *Escherichia coli* 555 metabolome by using 1H-NMR spectroscopy. Food Chemistry, 141(4), 4367-4374.
- ✓ Pisacane V., Callegari M.L., Puglisi E., Dallolio G., Rebecchi A. (2015). Microbial analyses of traditional Italian salami reveal microorganisms transfer from the natural casing to the meat matrix. *International Journal of Food Microbiology* 207: 57-65;
- ✓ R Core Team. (2018). R: A Language and Environment for Statistical Computing. Vienna, Austria. Retrieved from https://www.r-project.org/

- ✓ Rantsiou K. and Cocolin L. (2006). New developments in the study of the microbiota of naturally fermented sausages as determined by molecular methods: A review. *International Journal of Food Microbiology* 108: 255-267;
- ✓ Ravyts F., Steen L., Goemaere O., Paelinck H., De Vuyst L., Leroy F. (2010). The application of staphylococci with flavor generating potential is affected by acidification in fermented dry sausages. *Food Microbiology*, 27: 945-954.
- ✓ Ravyts F., De Vuyst L., Leroy F. (2012). Bacterial diversity and functionalities in food fermentations. *Engineering in Life Sciences*,12, 1-12.
- ✓ Rimaux, T., Vrancken, G., Pothakos, V., Maes, D., De Vuyst, L., and Leroy, F. (2011a). The kinetics of the arginine deiminase pathway in the meat starter culture Lactobacillus sakei CTC 494 are pH-dependent. *Food Microbiology* 28, 597-604.
- ✓ Rimaux, T., Vrancken, G., Vuylsteke, B., De Vuyst, L., Leroy, F. (2011b). The pentose moiety of adenosine and inosine is an important energy source for the fermented-meat starter culture *Lactobacillus sakei* CTC 494. Applied and *Environmental Microbiology* 77, 6539-6550.
- ✓ Rimaux, T., Riviére, A., Illeghems, K., Weckx, S., De Vuyst, L., Leroy, F. (2012). Expression of the arginine deiminase pathway genes in *Lactobacillus sakei* is strain-dependent and is affected by environmental pH. Applied and Environmental Microbiology, 78, 4874–4883.
- ✓ Rodel W., Stiebing A., Krockel L. (1993). Ripening parameters for traditional dry sausages with a mould covering. *Fleisch-wirtschaft*, 73: 848-853;
- ✓ Rodriguez-Jerez J.J., Colavita G., Giaccone V., Parisi E. (1994a). *Bacillus macerans*, a new potential histamine producing bacteria isolated from Italian cheese. *Food Microbiology* 11: 409-415;
- ✓ Roncales P. (2007). Additives. In F. Toldrà (Ed.), *Handbook of fermented meat and poultry* (pp. 77-86). Ames, Iowa, USA: Blackwell Publishing;
- ✓ Rousseau S. (2012). Food representations. In A. Bentley (Ed.), *A cultural history of food in the modern age* (pp. 183-200). London & New York: Berg Publishers;
- ✓ Ruiter A. (1979). Color of smoked foods. *Food Technology* (5): 54–63;

- ✓ Ruiz-Ramirez J., Serra X., Arnau J., Gou P. (2005). Relationship between water content, NaCl content, pH and texture parameters in dry-cured muscles. *Meat Science* 70: 579;
- ✓ Salvetti, E., Harris, H.M.B., Felis, G.E., O'Toole, P.W. (2018). Comparative genomics of the genus *Lactobacillus* reveals robust phylogroups that provide the basis for reclassification. Applied and Environmental Microbiology, 84, e00993-18.
- ✓ Samartzidou H., Delcour A.H. (1999). Excretion of endogenous cadaverine leads to a decrease in porin – mediated outer membrane permeability. *Journal of Bacteriology* 181, 791 – 798;
- ✓ Samelis J., Metaxopoulos J., Vlassi M. and Pappa A. (1998). Stability and safety of traditional Greek salami-A microbiological ecology study. *International Journal of Food Microbiology* 44: 69-82;
- ✓ Sánchez-Mainar, M., Stavropoulou, D.A., Leroy, F. (2017). Exploring the metabolic heterogeneity of coagulase-negative staphylococci to improve the quality and safety of fermented meats: a review. International Journal of Food Microbiology, 247, 24-37.
- ✓ Santchurn S.J., Collignan A. (2007). Fermented poultry sausages. In F. Toldrà (Ed.), *Handbook of fermented meat and poultry* (pp.361-368). Ames, Iowa, USA: Blackwell Publishing;
- ✓ Santos C., Jalon M., Marine A. (1985). Contenido de tiramina en alimentoa de origen animal. I. came. derivados carnicos y productos relacionados. *Revista de Agroquímica y Tecnología de Alimentos* 25, 362-368;
- ✓ Sanz B., Selgas D., Parejo I. and Ordonez J.A. (1998). Characteristics of lactobacilli isolated from dry fermented sausages. *International Journal of Food Microbiology* 6: 199-205;
- ✓ Sanz Y., Fadda S., Vignolo G., Aristoy C., Oliver G. and Toldrá F. (1999). Hydrolysis of muscle myofibrillar proteins by *Lactobacillus curvatus* and *Lactobacillus sakei*. *International Journal of Food Microbiology* 53: 115-125;
- ✓ Sanz Y., Tolda F. (2001). Purification and charactzerization of an Xprolyl-dipeptidyl peptidase from *Lactobacillus sakei*. *Applied and Environmental Microbiology*, 67:1815-1820;

- ✓ Schillinger U., Luècke F.K. (1989). Einsatz von Milchsaur-ebakterien als Schutzkulturen bei Fleischerzeugnissen. *Fleisch-wirtschaft*, 69: 879-882;
- ✓ Schmidt S., Berger R.G. (1998). Microbially formed aroma compounds during the maturation of dry fermented sausage (salami). *Advances in Food Sciences*, 20: 144-152.
- ✓ Schmidt G., Hertel C., Hammes W.P. (1999). Molecular characterisation of the dnaK operon of *Lactobacillus sakei* LTH681. *Systematic and Applied Microbiology* 22: 321-328.
- ✓ Shahidi F., Pegg R.B., Sen N.P. (1994). Absence of volatile N – nitrosoamines in cooked nitrite – free cured muscle food. *Meat Science* 37, 327 – 336;
- ✓ Shalaby A. (1996). Significance of biogenic amines to food safety and human health. *Food Research International* 29 (7): 675-690;
- ✓ Shibata, N., and Toraya, T. (2015). Molecular architectures and functions of radical enzymes and their (re)activating proteins. J. Biochem. 158, 271-292.
- ✓ Silla Santos M.H. (1996). Biogenic amines: their importance in food. *International journal Food Microbiology* 29, 213-231;
- ✓ Simon L., Fremaux C., Cenatiempo Y., Berjeaud J.M., (2002). Sakacin G, a new type of antilisterial bacteriocin. *Applied and Environmental Microbiology*, 68:6416-6420;
- ✓ Sims G.G., Fam G., York R.K. (1992). Quality indices for canned Skipjack tuna: correlation of sensory attributes with chemical indices. *Journal of Food Science* 57, 1112- 1115;
- ✓ Sindelar J.J., Milkowski A.L. (2012). Human safety controversies surrounding nitrate and nitrite in the diet. *Nitric Oxide*, 15: 259-266;
- ✓ Sinz, Q., and Schwab, W. (2012). Metabolism of amino acids, dipeptides and tetrapeptides by *Lactobacillus sakei*. Food Microbiol. 29, 215-223.
- ✓ Sinz, Q., Freiding, S., Vogel, R.F., and Schwaba, W. (2013). A hydrolase from *Lactobacillus sakei* moonlights as a transaminase. Appl. Env. Microbiol. 79, 2284-2293.
- ✓ Skandamis P. and Nychas G.-J. E. (2007). Pathogens: Risks and control. In *Handbook of Fermented Meat and Poultry*, edited by Toldra F., Hui Y.H., Astiasaran

I., Nip W.-K., Sebranek J.G., Silveira E.-T.F., Stahnke L.H. and Talon R. Oxford, U.K.: Blackwell Publishing;

- ✓ Skeie S., Kieronczyk A., Næss R. M., and Østlie H. (2008). *Lactobacillus* adjuncts in cheese: their influence on the degradation of citrate and serine during ripening of a washed curd cheese. *International Dairy Journal*, 18:158-168;
- ✓ Smacchi E., Gobbetti M. (1998). Purification and characterization of cystathionine g-lyase from *Lactobacillus fermentum* DT41. FEMS *Microbiology Letters*, 166:197-202;
- ✓ Smit, B.A., Engels, W.J.M., Wouters, J.T.M., Smit, G. (2004). Diversity of L-leucine catabolism in various microorganisms involved in dairy fermentations, and identification of the rate-controlling step in the formation of the potent flavor component 3-methylbutanal. Applied and Microbiological Biotechnology, 64, 396-402.
- ✓ Smid E.J., Kleerebezem M. (2014). Production of aroma compounds in lactic fermentations. *Annual Review Food Science and Technology*, 5:313-326.
- ✓ Sonjak S., Ličen M., Frisvad J.C., Gunde-Cimerman N. (2011). The mycobiota of three dry-cured meat products from Slovenia. Food Microbiology 28 (3): 373-376;
- ✓ Speranza, G., Corti, S., Fontana, G., Manitto, P. (1997). Conversion of *meso*-2,3-butanediol into 2-butanol by Lactobacilli. Stereochemical and enzymatic aspects. Journal of Agricultural and Food Chemistry, 45, 3476-3480.
- ✓ Stahnke L.H. (1995). Dried sausages fermented with Staphylococcus xylosus at different temperatures and with different ingredient levels. III. Sensory evaluation. *Meat Science*, 41: 211-223;
- ✓ Stahnke H., Holck A., Jensen A., Nilsen A. and Zanardi E. (2002). Maturity acceleration of Italian dried sausage by *Staphylococcus carnosus* - relationship between maturity and flavor compounds. *Journal of Food Science* 67: 1914-1921;
- ✓ Stavropoulou, D.A., Borremans, W., De Vuyst, L., De Smet, S., Leroy, F. (2015). Amino acid conversions by coagulase-negative staphylococci in a rich medium: Assessment of inter- and intraspecies heterogeneity. International Journal of Food Microbiology 212 Stahnke, L.H., 1995b. Dried sausages fermented with *Staphylococcus xylosus* at different temperatures and with different ingredient levels

— part III. sensory evaluation. Meat Science, 41, 211-223.

- ✓ Stentz R., Zagorec M. (1999). Ribose utilisation in *Lactobacillus sakei*: analysis of the regulation of the rbs operon and putative involvement of a new transporter. *Journal of Molecular Microbiology and Biotechnology* 1: 165-173;
- ✓ Stratton J. E., Hutkins R. W., e Taylor S. L. (1991). Biogenic amines in cheese and other fermented foods: A review. *Journal of Food Protection* 54, 460–470;
- ✓ Sunesen L.O., Stahnke L.H. (2003). Mould starter cultures for dry sausages-selection, application and effects. *Meat Science* 65 (3): 935-948;
- ✓ Suzzi G. and Gardini F. (2003). Biogenic amines in dry fermented sausages: A review. *International Journal of Food Microbiology* 88: 41-54;
- ✓ Tabanelli, G., Coloretti, F. Chiavari, C., Grazia, L., Lanciotti, R., Gardini, F. (2012). Effects of starter cultures and fermentation climate on the properties of two types of typical Italian dry fermented sausages produced under industrial conditions. Food Control, 26, 416-426.
- ✓ Talón D., Deliere E. and Bertrand X. (2002). Characterization of methicillin-resistant *Staphylococcus aureus* strains susceptible to tobramycin. *International Journal of Antimicrobial Agents* 20 (3): 174-179;
- ✓ Talón R., Leroy S., Lebert I., Giammarinaro P., Chacornac J., Latorre-Moratalla M., Vidal-Carou C., Zanardi E., Conter M. and Lebecque A. (2008). Safety improvement and preservation of typical sensory qualities of traditional dry fermented sausages using autochthonous starter cultures. *International Journal of Food Microbiology* 15-126 (1-2): 227-34;
- ✓ Ten Brink B., Damink C., Joosten H.M.L.J., e Huis in‘t Veld J.H.J. (1990). Occurrence and formation of biologically active amines in foods. *International Journal of Food Microbiology* 11: 73-84;
- ✓ Teodorovic V., Buncic S., Smiljanic D. (1994). A study of factors influencing histamine production in meat. *Fleischwirtschaft* 74, 170–172.
- ✓ Tiecco G., Tantillo G., Francioso E., Paparella A., De Natale G. (1986). Ricerca quali-quantitativa di alcune amine biogene in insaccati nel corso della stagionatura. *Industrie Alimentari* 5: 209-213;

- ✓ Tkachenko A.G., Nesterova L.Y., Pshenichnov K. (2001). Role of putrescine in the regulation of the expression of the oxidative stress defence genes of *Escherichia coli*. *Microbiology* 70, 133 – 137;
- ✓ Toldrá F. (2006). The role of muscle enzymes in dry-cured meat products with different drying conditions. *Trends in Food Science and Technology* 17: 164;
- ✓ Torriani, S., Van Reenen, G.A., Klein, G., Reuter, G., Dellaglio, F., and Dicks, L.M. (1996). *Lactobacillus curvatus* subsp. *curvatus* subsp. *nov*. and *Lactobacillus curvatus* subsp. *melibiosus* subsp. *nov*. and *Lactobacillus sake* subsp. *sake* subsp. *nov*. and *Lactobacillus sake* subsp. *carnosus* subsp. *nov*., new subspecies of *Lactobacillus curvatus* Abo-Elnaga and Kandler 1965 and *Lactobacillus sake* Katagiri, Kitahara, and Fukami 1934 (Klein et al. 1996, emended descriptions), respectively. Int. J. Syst. Bacteriol. 46, 1158-1163;
- ✓ Tóth L., Potthast K. (1984). Chemical aspects of the smoking of meat and meat products. In *Advances in Food Research*, vol.29, edited by C.O. Chichester, E.M.K. Mrak, and B.S. Schweigert. New York: Academic Press;
- ✓ Tremonte, P., Reale, A., Coppola, R., Succi, M. (2005). Preliminary investigations on microbiological characteristics of "ventricina". Industrie Alimentari, 44, 753-757.
- ✓ Tremonte, P., Sorrentino, E., Pannella, G., Tipaldi, L., Sturchio, M., Masucci, A., Maiuro, L., Coppola, R., Succi, M. (2017). Detection of different microenvironments and *Lactobacillus sakei* biotypes in Ventricina, a traditional fermented sausage from central Italy. International Journal of Food Microbiology, 242, 132-140.
- ✓ Troy D.J., Kerry J.P. (2011). Consumer perception and the role of science in the meat industry. *Meat Science*, 86: 214-226;
- ✓ Urso R., Comi G. and Cocolin L. (2006a). Ecology of lactic acid bacteria in Italian fermented sausages: Isolation, identification and molecular characterization. *Systematic Applied Microbiology* 29: 671-680;
- ✓ Urso R., Rantsiou K., Cantoni C., Comi G., Cocolin L. (2006b). Technological characterization of a bacteriocin-producing *Lactobacillus sakei* and its use in fermented sausages production. *International Journal of Food Microbiology*, 110:232-239;

- ✓ Van den Berg D., Robijn G.W., Janssen A.C., Giuseppin M.L.F., Kamerling J.P., Vliegenhart J.F.G., Ledeboer A.M., Verrips C.T. (1995). Production of a novel extracellular polysaccharide by *Lactobacillus sake* 0-1 and characterization of the polysaccharide. *Applied and Environmental Microbiology*. 61: 2840-2844;
- ✓ Veciana M.T., Vidal M.C., Marine A. (1989). Histamine and tyramine during storage and spoilage of anchovies *Engraulis encrasichohu*: relationships with other fish spoilage indicators. *Journal of Food Science* 55, 1192-l 195.
- ✓ Vidal-Carou M.C., Izquierdo-Pulido M.L., Martin-Morro M.C., Marine-Font A. (1990). Histamine and tyramine in meat products: relationship with meat spoilage. Food Chemistry 37, 239–249;
- ✓ Vinci G., Antonelli M.L. (2002). Biogenic amine quality index of freshness in red and white meat. Food Control 13, 519–524;
- ✓ Visagie C.M., Houbraken J., Frisvad J.C., Hong S.B., Klaassen C.H.W., Perrone G., Seifert K.A., Varga J., Yaguchi T., Samson R.A. (2014). Identification and nomenclature of the genus *Penicillium*. *Study in Mycology* 78: 343-371;
- ✓ Vogel R.F., Pohle B.S., Tichaczek P.S., Hammes W.P. (1993). The competitive advance of *Lactobacillus curvatus* LTH 1174 in sausage fermentations is caused by formation of curvacin A. *Systematic and Applied Microbiology*, 16: 457-462;
- ✓ von Wright, A., Axelsson, L. (2011). Lactic acid bacteria: an introduction. In *Lactic Acid Bacteria, Microbial and Functional Aspects, 4th Edn*, eds S. Lahtinen, A.C. Ouwehand, S. Salminen, A. von Wright, 1-16. Boca Raton: CRC Press.
- ✓ Watts B.M., Faulkner M. (1954). Antioxidative effects of liquid smokes. *Food Technology* 8:158-161;
- ✓ West H. (2008). Food fears and raw-milk cheese. *Appetite*, 51: 25-29;
- ✓ Xu, H., Gao, L., Jiang, Y., Tian, Y., Peng, J., Xa, Q., et al. (2015). Transcriptome response of *Lactobacillus sakei* to meat protein environment. J. Basic Microbiol. 55, 490-499;
- ✓ Yankah V.V., Ohshima T., Koizumi C. (1993). Effects of processing and storage on some chemical characteristics and lipid composition of a Ghanaian fermented fish product. Journal of the Science of Food and Agriculture 63, 227-235.

- ✓ Yildiz-Turp G., Serdaroglu M. (2008). Effect of replacing beef fat with hazelnut oil on quality characteristics of sucuk e a Turkish fermented sausage. *Meat Science*, 78: 447-454;
- ✓ Zagorec M., Champomier-Vergès M.C. (2017). *Lactobacillus sakei*: A starter for sausage fermentation, a protective culture for meat products. *Microorganisms*, 5:56;
- ✓ Zanardi E., Ghidini S., Conter M., Ianieri A. (2010). Mineral composition of Italian salami and effect of NaCl partial replacement on compositional, physico-chemical and sensory parameters. *Meat Science*, 86: 742-747;
- ✓ Zdolec N., Kozačinski L., Hadžiosmanović M., Cvrtila Z., Filipović I., (2007). Inhibition of *Listeria monocytogenes* growth in dry fermented sausages. *Veterinarski Arhiv*, 77:507-514;
- ✓ Zeuthen P. (2007). A historical perspective of meat fermentation. In Toldrà F. (Ed.), *Handbook of fermented meat and poultry* (pp. 3-8). Ames, Iowa, USA: Blackwell Publishing;
- ✓ Zhang, Y., Dai, Z., Krivoruchko, A., Chen, Y., Siewers, V., and Nielsen, J. (2015). Functional pyruvate formate lyase pathway expressed with two different electron donors in *Saccharomyces cerevisiae* at aerobic growth. FEMS Yeast Res. 15, 1-8;
- ✓ Zhu, C., Li, C., Wang, Y., Laghi, L. (2019). Characterization of Yak Common Biofluids Metabolome by Means of Proton Nuclear Magnetic Resonance Spectroscopy. Metabolites. 9(3): 41.
- ✓ Zwietering, M.H., Jongenburger, I., Rombouts, F.M., and van't Riet, K. (1990). Modeling of the bacterial growth curve. Applied and Environmental Microbiology, 56, 1875-1881.